FLORIDOSIDES IN *PORPHYRA PERFORATA*

by

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Floridosides in Porphyra perforata

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ABSTRACT

The chemical structures and configurations of floridoside (sn-2'-O-α-D-galactopyranosyl glycerol) and isofloridoside, D-form (sn-3'-O-α-D-galactopyranosyl glycerol) and L-form (sn-1'-O-α-D-galactopyranosyl glycerol), obtained by extraction of the red alga *Porphyra perforata*, were studied using nuclear magnetic resonance spectroscopy (NMR) and gas-liquid chromatography-mass spectroscopy (GLC-MS). Separation and quantitation of floridoside and D- and L-forms of isofloridoside can be achieved by a combination of GLC-MS, and ^{1}H- and ^{13}C-NMR. The ratio of D- to L-forms of isofloridoside varied in the samples analyzed.

Methods for extraction and assay of floridoside phosphate synthase (FPS, UDP-galactose: sn-3-glycerol phosphate 1->2' α-D-galactosyl transferase) were developed and FPS was partially purified and characterized. Two assay methods were developed, one measuring the formation of floridoside phosphate, the other measuring sn-3-glycerol phosphate dependent formation of UDP. FPS is a soluble protein and has a high substrate specificity, only UDP-galactose and sn-3-glycerol phosphate can be used as substrates. The FPS activity was stable in concentrated solutions (in 0.3 M ammonium sulfate), the reaction was linear up to 45 min, and proportional to protein concentration in the incubation mixture. The enzyme activity increased with temperature from 0°C to 35°C and was lost above 40°C.

In field samples, the level of FPS activity varied with time of day, and floridoside content increased about three fold 3 h after sunrise. This level dropped gradually in the afternoon, and remained relatively stable overnight.

FPS was purified 27-fold by DEAE-ion exchange and HPLC ion-exchange chromatography. Its molecular weight, as determined by HPLC gel-filtration, was about 140 kD. Hyperbolic kinetics were obtained with increasing concentrations of uridine diphosphate galactose (UDP-GAL) and sn-3-glycerol phosphate (GLYP). The apparent K_m values for these substrates were 3, and 10 mM, respectively. Various amino acids, organic acids, nucleotides, each at a concentration of 5 mM, had no effect on the activity of the enzyme, but one of the reaction products, UDP, inhibited the activity. These results suggest that the activity of FPS *in situ* may be controlled by the intracellular UDP-GAL:UDP ratio.

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To Kun Shao, my wife, her patience and encouragements were invaluable. This thesis is dedicated to my mother.

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Bes	(N,N-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid)
BSA	Bovine serum albumin
DTT	Dithiothreitol
EDTA	Ethylenediamine tetraacetate
FPS	Floridoside phosphate synthase
GAL	Galactose
GLC	Gas-liquid chromatography
GLC-MS	Gas-liquid chromatography-mass spectroscopy
HPLC	High performance liquid chromatography
IR	Infrared spectroscopy
GLYP	sn-3-glycerol phosphate
LN_2	Liquid nitrogen
NMR	Nuclear magnetic resonance spectroscopy
PC	Paper chromatography
Pi	Inorganic phosphate
PMSF	Phenylmethylsulfonyl fluoride
ppm	Part per million
pptd	Precipitated
PVPP	Polyvinylpolypyrrolidone
RuBP	Ribulose 1,5-bisphosphate
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SPS	Sucrose phosphate synthase
TLC	Thin layer chromatography

TMS	Trimethylsilyl
Tricine	N-Tris(hydroxymethyl)methylglycine
UDP-GAL	Uridine diphosphate galactose

CHAPTER 1

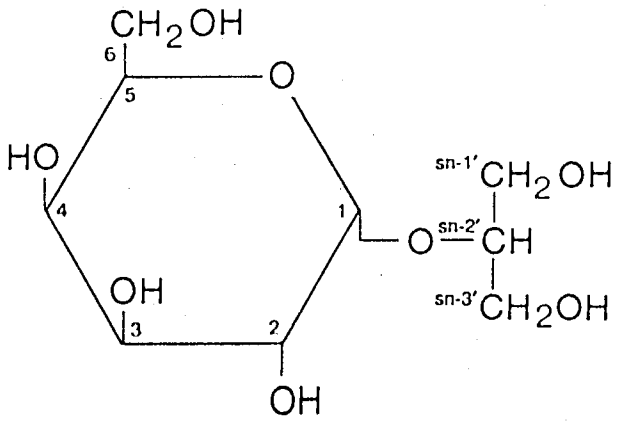
INTRODUCTION

All plants carrying out photosynthesis produce ATP and reduced NADP from photochemical reactions. This chemical energy is in turn stored in carbohydrates by the fixation and subsequent reduction of CO_2. Carbohydrates are important to the plant in several ways. First, they provide energy via degradation for other cellular functions. Second, they themselves are important constituents of the cell. Third, they provide the carbon atoms for the biosynthesis of organic compounds present in the plant.

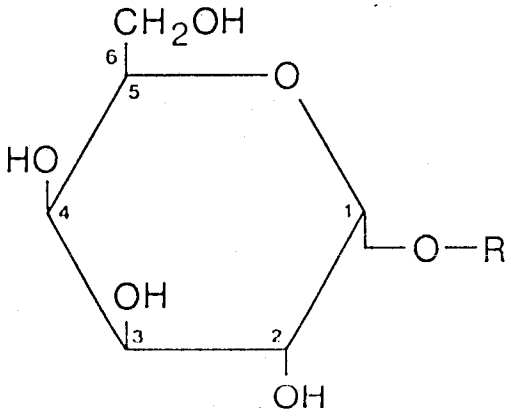
Floridoside, (sn-2'-O-α-D-galactopyranosyl glycerol) is one of the major low molecular weight carbohydrate derivatives in red algae except for those belonging to the order Ceramiales (Craigie *et al.* 1968; and Reed *et al.* 1980b). It is also the major photosynthetic product in these algae (Bean and Hassid 1955, Bidwell 1958, Craigie *et al.* 1968, and Nagashima *et al.* 1969) and its concentration *in vivo* has been shown to respond to a wide range of environmental conditions. But, little is known about floridoside production and degradation, and control mechanisms in these processes.

In the order Bangiales, another low molecular weight carbohydrate derivative, isofloridoside is also present and it can occur in the D-form (sn-3'-O-α-D-galactopyranosyl glycerol) or the L-form (sn-1'-O-α-D-galactopyranosyl glycerol). The physiological function of isofloridoside is still largely unknown.

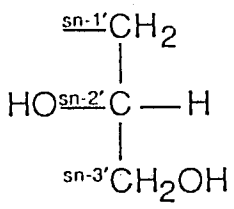
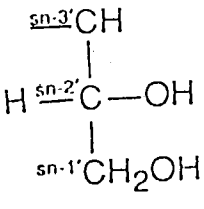
Fig. 1. The structures of floridoside (sn-2'-O-α-D-galactopyranosyl glycerol) and isofloridoside, D-form (sn-3'-O-α-D-galactopyranosyl glycerol) and L-form (sn-1'-O-α-D-galactopyranosyl glycerol).



Floridoside



Isofloridoside

R =  

L- Form D - Form

There has been some confusion and debate about these carbohydrates in *Porphyra*, and particularly whether isofloridoside occurs in the D- or the L-form or as a mixture of the D- and L-isomers. Because of the structural similarity between floridoside and isofloridoside, and the complexity added by D- and L-forms of isofloridoside, a thorough chemical analysis and establishment of separation methods for the various isomers of floridoside are required.

A few investigators have examined the changes in floridoside content in response to changes in external conditions. It has been proposed that floridoside functions similarly to sucrose in higher plants. However, unlike sucrose, the metabolic role(s) of floridoside and its relation to physiological processes in red algae are much less understood. Compared to sucrose in higher plants, there appear to be a few important differences in floridoside metabolism and function:

1. The existence of translocation in red algae has not been confirmed[1].
2. *Porphyra* is an intertidal plant, subject to frequent changes in salinity. Accordingly, it is of interest that floridoside was demonstrated to function directly in osmotic regulation.
3. The interactions of carbohydrates in *Porphyra* are likely to be different from those in higher plants: (1) the biosynthesis of floridean starch, a highly branched glucan containing α-1,4-

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[1]Hartmann and Eschrich (1969) reported translocation along midrib of a red alga *Delesseria sanguinea*. However, translocation has not been confirmed in other red algae (Meng and Wu, unpublished data; Floch, personal communication).

and α-1,6-glucosyl linkages, is reported to occur in the cytoplasm in red algae (Meeuse 1962), and (2) sucrose hydrolysis yields glucose and fructose, whereas the hydrolysis of floridoside yields galactose and glycerol. A few extra steps of isomerization of galactose are required for synthesis of floridean starch.

The metabolic pathways in *Porphyra* are still poorly understood. The key enzyme for the biosynthesis of floridoside, floridoside phosphate synthase (FPS), has not been studied. The key substrate, UDP-GAL could be used for either synthesis of floridoside or synthesis of polysaccharides. It is also interconvertible with UDP-glucose, which after been metabolized to ADP-glucose can be used for the biosynthesis of floridean starch. It is natural to assume that there must be some controlling points to regulate and coordinate all these processes, but there is no information on these control points.

In this thesis, I report on my investigations on floridosides in *Porphyra perforata*. Particular attention was given to:

1. Chemical characterization of floridosides.
2. Establishment of extraction and assay methods for FPS.
3. Field studies on floridoside content and changes in FPS activity.
4. Partial purification and characterization of FPS.

This topic was chosen for two reasons:

1. Floridoside is the major photosynthetic product in most red

algae. A study of FPS is expected to provide a framework for future studies on other major metabolic processes.

2. *Porphyra* is the most commonly consumed seaweed for food in China and Japan. It has a very high protein and carbohydrate content, and floridosides are believed to contribute to its good taste.

CHAPTER 2

LITERATURE REVIEW

I. LOW MOLECULAR WEIGHT CARBOHYDRATES IN *PORPHYRA*

In *Porphyra*, isofloridoside amounts to between 2.5 and 10.8 % of the dry weight and floridoside accounts for 0.8 to 6.1 % (McLachlan *et al*. 1972).

Floridoside was first detected by Kylin in 1918. Colin and co-workers demonstrated that floridoside occurs in many species of red algae (Colin and Guéguen 1930, Colin and Augier 1933). They isolated the compound and showed that it can be hydrolyzed with α-galactosidase, yielding equimolar quantities of D-galactose and glycerol. From this result, it was concluded that the galactoside possessed an α-linkage. Colin (1937) also showed that the galactoside was oxidized with difficulty by bromine, and that it was not attacked by *Acetobacter* (a bacterium, which is capable of oxidizing secondary hydroxyl, adjacent to the primary group, to a keto group); whereas the hydrolysis products were readily oxidized when treated with bromine or when inoculated with *Acetobacter*. On the basis of these results he concluded that the galactosidic linkage in the compound was through the secondary alcohol group of the glycerol. He proposed the structure of floridoside as 2-O-α-D-galactopyranosylglycerol (Fig. 1).

Colin's data did not afford a conclusive proof; accordingly, Putman and Hassid (1954) further investigated the structure of floridoside using methylation and periodate oxidation procedures. From the methylation and subsequent hydrolysis,

2,3,4,6-tetra-O-methyl-D-galactose and 1,3-di-O-methylglycerol were obtained, indicating a linkage through position 2 of glycerol. Additional evidence for the galactosylglycerol structure was provided from periodate oxidation of floridoside. On treatment of floridoside with sodium periodate, two moles of periodate were consumed and one mole of formic acid produced. No formaldehyde could be found in the reaction mixture. These data were consistent only with a structure in which the D-galactopyranosyl moiety was linked to the secondary alcohol of glycerol. Thus the structure of floridoside as originally proposed by Colin (1937) was confirmed.

Isofloridoside was first detected by Lindberg (1955) during his studies of red algae from the order Bangiales. He deduced its α-galactosidic structure by its high optical rotation. However, since there is another chiral center on glycerol moiety, high optical rotation does not unequivocally indicate an α-linkage. The products of hydrolysis, glycerol and galactose, suggested that it was isomeric with floridoside. On the basis of these data, Lindberg proposed the structure for isofloridoside as 1-glycerol α-galactopyranoside. It is well established that in both floridoside and isofloridoside, the galactose is in the D-configuration (Putman and Hassid, 1954; Wickberg, 1958). Even though the configuration of the glycerol part of isofloridoside was not investigated, Lindberg (1955) correctly pointed out that the glycerol moiety has a chiral center and thus there are two isomeric forms of isofloridoside. Wickberg (1958) synthesized D-

and L-forms of isofloridoside from glycerol derivatives of known configuration and provided melting point and infra-red spectra for each isomer.

There was some disagreement about the form in which isofloridoside naturally occurs in *Porphyra*. Wickberg (1958) reported that isofloridoside occurred as an isomorphous D- and L-mixture, which was confirmed by Craigie *et al.* (1968). In contrast Peat and Rees (1961), and Su and Hassid (1962) reported that isofloridoside occurred as the pure D-form. This difference was attributed to different *Porphyra* species being analyzed (Su & Hassid, 1962).

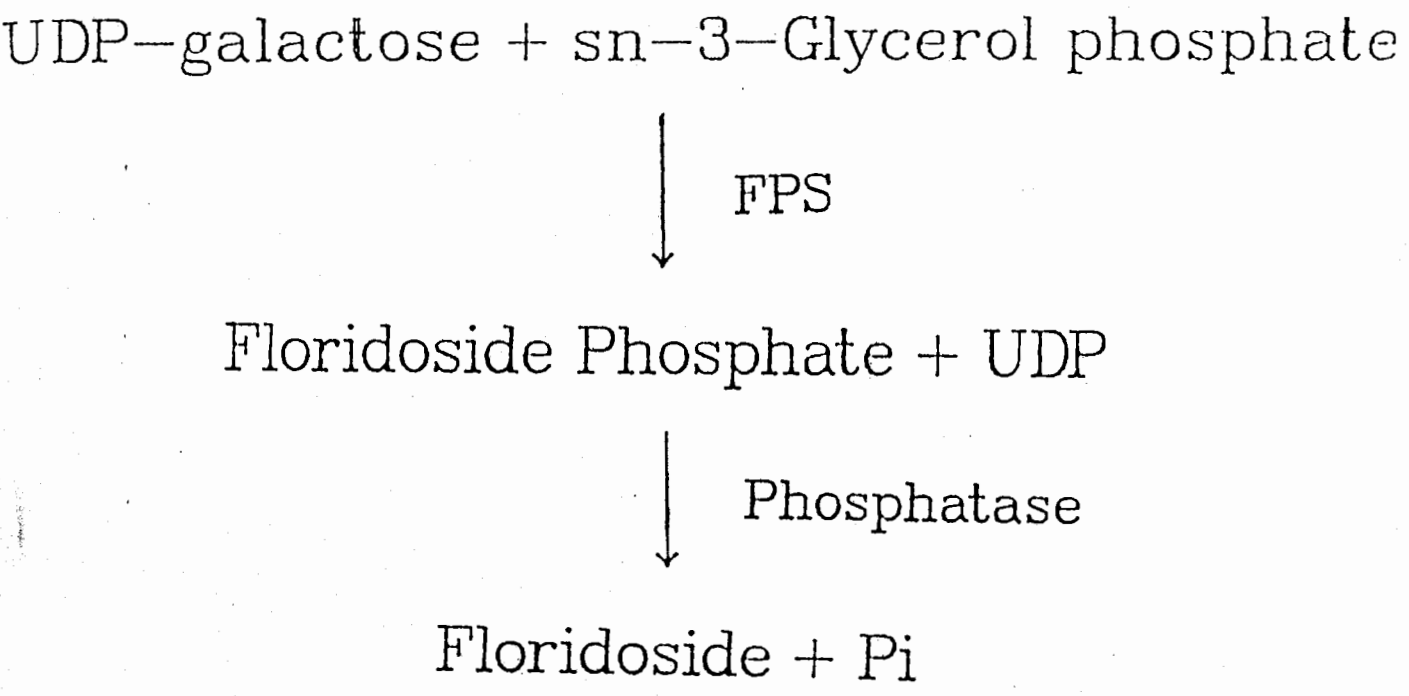
The structure of floridoside has been confirmed by gas-liquid chromatography-mass spectroscopy (GLC-MS) (Reed *et al.* 1980b, and Nagashima and Fukuda 1983), ^{1}H-nuclear magnetic resonance spectroscopy (^{1}H-NMR) (Alpin *et al.* 1967), and recently by ^{13}C-NMR (Van Der Kaaden *et al.* 1984), but few data are published with regard to the structure of isofloridoside. No satisfactory method for the separation of D- and L-forms of isofloridoside has been reported. Wickberg (1958) used a column packed with activated carbon particles to resolve two isomers of isofloridoside, but the separation was incomplete and time-consuming and the column is apparently unsuitable for analytical purposes. Although GLC-MS analysis of pertrimethylsilylated isofloridoside has been published (Nagashima & Fukuda 1983) and Beier *et al.* (1980) assigned part of ^{13}C-NMR spectrum of isofloridoside, they did not distinguish

between D- and L-forms of isofloridoside.

II. SYNTHESIS OF FLORIDOSIDE

The biosynthetic pathway for floridoside was first proposed by Bean and Hassid (1955). They fed $^{14}CO_2$ to a photosynthesizing red alga *Iridophycus flaccidum* (=*Iridaea flaccida*) for periods ranging from 8 seconds to 24 h, and the resulting radioactive components were separated and identified by paper chromatographic and radioautographic techniques. Analysis of the rates of fixation of ^{14}C (from $^{14}CO_2$) in the various compounds served to indicate their probable position in the sequence of reactions leading to the formation of floridoside. It was found that in the period between 8 and 15 seconds, ^{14}C-labeled glycerol 1-phosphate and UDP-glucose appeared. Between 15 and 30 seconds of photosynthesis, radioactive UDP-galactose was detected. The labeled floridoside appeared during the same period. From these data, they proposed a pathway for the biosynthesis of floridoside in red algae. The synthesis involves the transfer of a galactose from a donor, uridine diphosphate-galactose (UDP-GAL), to an acceptor, sn-3-glycerol phosphate (GLYP). The product, floridoside phosphate (sn-2'-O-α-D-galactopyranosyl 1->2' glycerol phosphate), undergoes dephosphorylation to floridoside. It was also proposed that glycerol 1-phosphate is derived by the reduction of a triose phosphate, and UDP-GAL comes from UDP-glucose. Since most of the phosphorylated products formed in the earlier stages of photosynthesis of red algae appear to be identical with those produced in the green plants, the dark fixation reactions in the

Fig. 2. Scheme indicating pathway of floridoside biosynthesis.


UDP–galactose + sn–3–Glycerol phosphate
FPS
Floridoside Phosphate + UDP
Phosphatase
Floridoside + Pi

chloroplasts of red algae are probably similar to those in green plants. Kremer and Kirst (1981) subsequently confirmed these results of Bean and Hassid (1955) and, in addition, reported the occurrence of glycerol 1-phosphate dehydrogenase in marine red algae, which converted a triose phosphate, dihydroxyacetone phosphate, to glycerol 1-phosphate at the expense of NADH. Floridoside phosphate synthase activity was also detected in their extracts but at a very low level.

III. FLORIDOSIDE AS A PHOTOSYNTHETIC AND STORAGE COMPOUND

Numerous investigators have shown that floridoside is the major photosynthetic product in most red algae (Bean and Hassid 1955, Bidwell 1958, Majak *et al.* 1966, Craigie *et al.* 1968, Nagashima *et al.* 1969, and Macler 1986). Majak *et al.* (1966) studied photosynthetic rates and carbon partitioning of photosynthetic products in *Porphyra umbilicalis*. They found that *P. umbilicalis* had a photosynthetic rate of 90 μmol C h^{-1} g^{-1} fresh wt, and that 55% of total fixed carbon accumulated in floridoside. Considering that there are 9 carbon atoms in floridoside, the floridoside production rate *in vivo* will be 5.5 μmol h^{-1} g^{-1} fresh wt. In studying ^{14}C fixation by the red alga *Gelidium coulteri*, Macler (1986) found that in the light, ^{14}C labeling of floridoside did not level off over a 7 h period, but instead continued to increase throughout the experiment. In the following chase period, cultures showed large decreases in ^{14}C label in floridoside both in the light and in the dark. It was calculated that from 30 to 50% of the floridoside pool was labeled in one day, a fast turnover rate. Macler (1986) suggested that most likely floridoside provided some carbons for agar synthesis and for the continued synthesis of amino acids. ^{14}C incorporation in the dark was only 1 to 7% of that seen in the light. The percent of floridoside formed in the dark was low relative to that in the light. Nagashima *et al.* (1969) also reported that during a chase period in the dark following a ^{14}C-feeding experiment in light, the radioactivity of

floridoside remained constant whereas that in floridean starch, was markedly decreased. The feeding of ^{14}C-floridoside to plants revealed that floridoside and floridean starch are rapidly interconvertible in intact cells (Nagashima *et al.* 1969). Based on these results, it has been suggested that floridoside is a reserve carbohydrate analogous to sucrose in higher plants.

IV. REGULATION OF FLORIDOSIDE BIOSYNTHESIS

It has been shown that the *in vivo* level of floridoside varies in response to variations in salinity (Reed *et al*. 1980b, Wiencke and Läuchli 1981, and Macler 1988), or nitrogen level in the medium (Macler 1986). These changes may require an induction period of several hours at the altered conditions (Macler 1988). Since floridoside is a photosynthetic product, it would be expected that floridoside production will change with photosynthetic rates, light intensity, as well as light and dark periods. But not much work has been done on this aspect.

1. Salinity effects on floridoside level

Intertidal seaweeds are subject to marked changes in salinity. Fresh water from rain or stream flows may lower salinity; evaporation, when plants are exposed at low tide or trapped in tide pools, may raise salinity. Also, for submerged plants under estuarine conditions, tidal flows may cause large changes in salinity. Altered salinity levels result in changes in turgor in plants.

It is known that algae capable of osmotic regulation respond to changes in external osmotic potential by altering internal ion concentrations and/or by synthesis or degradation of low molecular weight organic compounds (Hellebust 1976, Gutknecht *et al*. 1978, Kauss 1978, Zimmermann 1978, Bisson and Kirst 1979,

and Kirst and Bisson 1979). Both strategies may be used together by the same organism. Ahmad and Hellebust (1984) indicated that microalgae at high salinities must have accumulated some inorganic ions in addition to the organic solutes for osmotic balance to exist between cells and the medium. It has been suggested that inorganic ions may play their major role as osmotically active agents in the vacuoles, whereas organic osmotic solutes may be restricted to the cytoplasm (Bisson and Kirst 1979, and Kirst and Bisson 1979). Such a model for cytoplasmic and vacuolar osmotic regulation has also been proposed for higher plants (Flowers *et al.* 1977, and Wyn Jones *et al.* 1977). But we cannot rule out the possibility of inorganic ions playing a role in the cytoplasm, as Ahmad and Hellebust (1984) pointed out that exclusion of external salt would be energetically very expensive to maintain and the cytoplasm of marine algae can tolerate the presence of a considerable amount of salt.

In *Porphyra purpurea*, turgor increases with decreasing salinity, while volume decreases as salinity increases in a passively regulated system (Reed *et al.* 1980a). Floridoside content was shown to respond to salinity changes. Reed *et al.* (1980b) showed increased floridoside levels with increased salinity and decreased levels with decreased salinity in *P. purpurea*. Wiencke and Läuchli (1981) also reported that the floridoside level increased immediately after onset of osmotic stress and could reach up to 4 X the value under normal

salinity. Their fine-structural studies suggested that floridoside was formed by hydrolysis of floridean starch granules at this stage (Wiencke and Läuchli, 1980). This assumption is supported by the earlier report by Nagashima *et al.* (1969) that floridoside and floridean starch are readily interconvertible. Changes in floridoside ^{14}C labeling pattern may not become obvious unless ^{14}C fixation experiments are carried out for several hours at the altered salinity, as Kremer (1979) was unable to detect changes in floridoside radioactivity level within the first hour. Reed *et al.* (1980b) reported that a period longer than 12 h was required for plants to reach a peak value of floridoside. During these salinity changes, isofloridoside level remained relatively constant. We do not know yet whether floridoside degradation is controlled in this process as well. In contrast to the situation in *Porphyra*, isofloridoside is the osmoregulator in *Ochromonas malhamensis*. Kauss (1978) suggested that changes in isofloridoside levels in response to salinity changes in *Ochromonas malhamensis* were a function of both synthesis and degradation of this compound.

2. Regulation of floridoside production by nitrogen

Macler (1986) reported that in the red alga *Gelidium coulteri* both the fixation rate of CO_2 and the flow of photosynthate into various end products were dependent on the nitrogen status of the tissue. Plants fed with high levels of nitrogen (330 μM NO_3^- and 6 μM NH_4^+) showed fixation rates

several-fold higher than those seen for plants starved for nitrogen. Also, nitrogen affected the partitioning of fixed carbon into floridoside, agar, and floridean starch. Plants under high nitrogen conditions had higher floridoside levels and markedly lower amounts of agar and starch than found in plants limited for nitrogen. Macler (1986) concluded that high levels of nitrogen stimulated amino acid synthesis at the expense of agar and starch. Even though no enzymological data were available, Macler (1986) speculated that a direct stimulation of FPS by nitrogen could explain increased floridoside production in nitrogen-enriched plants.

3. Enzymological regulation of floridoside production

In higher plants, the activity of sucrose phosphate synthase is highly regulated (Sicher and Kremer 1984, Brown and Huber 1987, and Stitt *et al.* 1988). It is known that sucrose increases during the initial hours of light and then remains at a constant level until darkness (Fondy and Geiger 1980, 1982, Sicher *et al.* 1984). It has been shown that SPS is regulated by glucose-6-phosphate and inorganic phosphate (Doehlert & Huber 1983). Similar data on the activity of floridoside phosphate synthase (FPS), the key metabolic enzyme for floridoside production in red algae, are not available. We also know very little about floridoside turnover rates and its pool sizes under various physiological conditions, or the control of interactions among major storage and structural carbohydrates in red algae.

There is no information about the site of biosynthesis of floridoside or cell wall polysaccharides in red algae. It is possible that nucleotide sugars required for these processes are present in the cytoplasm. Since floridean starch is synthesized in the cytoplasm (Meeuse 1962), it is possible that cell wall polysaccharides, floridean starch and floridoside all require nucleotide sugars from a common metabolic pool.

Only one prior attempt has been made to extract and assay for FPS activity. Kremer and Kirst (1981) used TLC to separate and quantify the floridoside phosphate formed by FPS-catalysis. Since they did not specify whether they used a standard (floridoside phosphate), it is difficult to check the authors' claim as to the identity and purity of a floridoside phosphate spot on TLC plate. No reliable method is available as yet to extract and assay floridoside phosphate synthase, and the enzyme has not been characterized.

The control of floridoside biosynthesis could be by one or a combination of the following mechanisms 1. Regulation of the rate by concentrations of substrates and products. 2. Regulation of the rate by ions and other metabolites (eg. via allosteric regulation). 3. Activation and inactivation of the enzyme (eg. via covalent modification, degradation, etc). All three levels of control have been shown or suggested for sucrose synthesis (Sicher and Kremer 1984, Brown and Huber 1987, and Stitt *et al.* 1988).

V. DISTRIBUTION OF FLORIDOSIDE IN A PLANT

In higher plants, sucrose is synthesized in the cytoplasm (Goodwin and Mercer 1983). In some plants, there may be two, interconvertible sucrose pools and these may be located in the cytoplasm and in the vacuole, respectively (Outlaw *et al.* 1975, and Sicher *et al.* 1984).

In red algae the compartmentation of floridoside is not clear. There is some indirect evidence that floridoside is located in the cytoplasm (Wiencke and Läuchli 1981). The concentration of floridoside and isofloridoside in *Porphyra* was greatest when plants were kept in media at 1.5 to 3 X normal salinity. When *Porphyra* plants were put in a medium at 4 X salinity, there was a strong depression of the intracellular floridoside and isofloridoside concentration in conjunction with a strong development of the vacuolar system, which suggests a nonvacuolar localization of floridoside. This evidence does not exclude the possibility of a minor floridoside pool in vacuoles.

VI. EXTRACTION OF PROTEINS FROM SEAWEED

Brown and red algal species contain high amounts of phenolic compounds and acidic polysaccharides (eg. alginic acid, carrageenan, and porphyran), which either interfere with protein extraction, or inactivate enzymes. There has been no systematic research on extraction of proteins from red algae, though a few papers have been published on the extraction and electrophoresis of proteins from brown algae (Marsden *et al.* 1981, Rice and Crowden 1987). There are large quantities of polyphenols in brown algae (9-14% dry matter) contained in membrane-limited vesicles (Evans and Holligan, 1972) which are ruptured during cell breakage. The polyphenols undergo progressive polymerization *in vivo* producing high molecular weight, non-dialyzable polyphloroglucinols (Ragan, 1976) which are readily oxidized on extraction (Crato, 1893a) to form low molecular weight phlorotannins (Koch *et al.* 1980). Tannins are well known to inhibit many enzymes (Crato 1893b, Ogino, 1962, Ohmann 1963, and Lin and Hassid 1966).

In most species of red algae, the majority of phenolic compounds are bromophenols, which occur predominantly as sulfate esters (Weinstein *et al.* 1975). However, the amount of bromophenols is low in the genera *Porphyra*, as Fenical (1975) did not find any significant formation of halogenated metabolites, including bromophenols in various species of *Porphyra*. Even in those red algal species which are known to

synthesize and accumulate bromophenols, the amount of bromphenols is still considerably less than the polyphenol content in brown algae. Ragan and Craigie (1978) reported that in *Polysiphonia lanosa (L.)* the hydrolyzed bromophenols sulfate were 2-3% of the dry weight, which is much less than 9-14% of dry weight for polyphenols in brown algae.

It would be expected that the low amounts of phenolic compounds in red algae will make extraction of proteins from red algae easier than that from brown algae, but denaturation of enzymes by exposure to low pH resulting from liberation of partially protonated acidic polysaccharides after cell disruption is also a potential, general problem. In *Porphyra*, an acidic polysaccharide, porphyran, is the principal component of cell walls and the intercellular matrix. Porphyran was found to consist of approximately 50% sulfated polysaccharides (Morrice *et al.* 1983). One way to avoid exposing proteins to extreme pH values is by using a high buffer concentration and homogenization in liquid nitrogen (LN_2). Grinding algal tissues in LN_2 prevents acidic polysaccharides from swelling and the inert environment restricts oxidation of phenols. With this technique many authors have successfully extracted proteins from brown algal tissues (Johnston and Davies 1969, Weidner *et al.* 1975, Kremer and Küppers, 1977, Küppers and Weidner, 1980, and Kerby and Evans, 1981).

Since most acidic polysaccharides will be charged in extraction buffer (close to neutral pH for most applications),

they may bind some extracted proteins. Murphy and Heocha (1973) suggested that a peroxidase from *Laminaria* was strongly bound to alginate. In addition, the presence of large amounts of viscous acidic polysaccharides in enzyme extracts renders subsequent purification and separation techniques difficult. It was found that anion exchange chromatography was an efficient way of removing acidic polysaccharides (Marsden *et al.* 1981). The vast majority of proteins were eluted by a salt gradient to 500 mM NaCl, the majority of polysaccharides were more tightly-bound and could only be eluted above 700 mM, whereas polyphenols and tannins remained bound to the column even at 1 M NaCl.

As mentioned earlier, Kremer and Kirst (1981) assayed FPS activity in species of red algae. They noticed that FPS was not stable in the crude extract, and that the formation of floridoside phosphate *in vitro* occurred at a much lower rate than *in vivo*. Apparently their extraction and/or the assay methods must be improved in order to get meaningful results.

CHAPTER 3

CHEMICAL CHARACTERIZATION OF FLORIDOSIDES FROM *PORPHYRA PERFORATA*

MATERIALS AND METHODS

1. Materials

Porphyra perforata J. Ag. was collected at various places along the coastline of Vancouver, B.C., Canada. Identification of *P. perforata* was determined by Drs. S. Lindstrom and M. Hawkes. One sample was taken from Friday Harbor, Washington, USA. The material was cleaned of visible epiphytes and epifauna and extracted immediately with 80% ethanol. The extract was concentrated to dryness on a rotary evaporator at a temperature below 40°C. The pigments and lipids were removed by extraction with chloroform. The residue was dissolved in water and desalted by either passing through Dowex 1(OH^-) and Dowex 50(H^+) ion-exchange resin columns (1.2 X 20 cm) or by adding an equal amount of these two resins to the sample solution while keeping the pH value at 7. The neutral fractions were evaporated, dissolved in absolute ethanol, and concentrated to a thick syrup. This material was used for separation of floridoside and isofloridoside using PC.

2. Methods

(1). Paper chromatography (PC)

Whatman No.1 and 3 papers were used for descending PC using the solvent system ethyl acetate - pyridine - water (8:2:1) and developed for 48 h (Nagashima and Fukuda, 1981). Floridoside and isofloridoside were detected by the periodate-benzidine reagent (Gordon *et al.* 1956). The R_f values were similar to those reported (Nagashima & Fukuda, 1981).

(2). Acetylation of floridosides

The samples and standards (5 mg) were each acetylated with pyridine-acetic anhydride (1:1, 1 mL) at 100°C for 1 h, concentrated to dryness, then dissolved in a small amount of ethyl acetate and analyzed by GLC-MS.

(3). Trimethylsilylation of floridosides

The dried samples (5 mg) were treated with "TRI-SIL 'Z'" (1 mL; Pierce Chemical Co.. Rockford, IL, USA) at 60-70°C for 1 h, with occasional sonication to enhance the dissolution of the samples. The resulting TMS derivatives were analyzed by GLC-MS.

(4). Methylation of floridosides

The floridosides (5 mg) were methylated with methyl iodide in the presence of methylsulfinyl anion according to the method of Hakomori (1964). The methylated products were recovered by partitioning between water and chloroform. The chloroform phase was concentrated to a small volume, and analyzed by GLC-MS. For

methylation analysis, the permethylated floridosides were treated with 90 % formic acid (2 mL) at 100°C for 2 h and then concentrated to dryness. This residue was further hydrolyzed with 0.5 M trifluoroacetic acid (2 mL) in an ampoule at 100°C for 16 h. The resulting partially methylated monosaccharides were analyzed as their alditol acetates, which were obtained after reduction with sodium borohydride and acetylation.

(5). Gas-liquid chromatography - mass spectrometry (GLC-MS)

GLC was performed on a Hewlett-Packard 5790A instrument, equipped with a flame ionization detector and connected to a model 3390A electronic integrator. A fused silica capillary column (SE-30, 12m X 0.2mm) was employed, with helium as carrier gas. Combined gas-liquid chromatography-mass spectrometry was carried out on a Hewlett-Packard 5985B GC/MS/DS using the above column and an ionization potential of 70 eV.

(6). Nuclear magnetic resonance spectroscopy (NMR)

NMR spectra were recorded at a probe temperature of 21°C on a Bruker WM-400 instrument operating in the pulsed Fourier transform mode with complete proton decoupling for ^{13}C-NMR. The chemical shifts are reported in parts per million (ppm) and related to internal acetone (δ=31.4 ppm for ^{13}C-NMR spectra, and δ=2.20 ppm for ^{1}H-NMR spectra). The samples used for ^{1}H-NMR were lyophilized three times with 99.7% D_2O and examined in the same solvent.

(7). Infrared spectroscopy (IR)

The samples were prepared as KBr discs and spectra were recorded with a Perkin-Elmer double beam spectrometer Model 599B at room temperature.

RESULTS

1. Extraction of floridosides

Extraction of *Porphyra perforata* with 80% ethanol and subsequent removal of pigments and lipids and desalting by passing through anion and cation exchange columns resulted in a neutral fraction suitable for analysis.

2. Purification of L-isofloridosides

From the above fraction, it was possible to obtain a compound by precipitation with acetone with a yield of 0.5%. This compound was recrystallized five times in equal amounts of ethanol and methanol and characterized as one form of isofloridoside by GLC-MS and NMR spectra. The melting point of this compound was 130.0-130.5°C and its infra-red spectrum was identical with that published for the L-form of isofloridoside (Wickberg 1958).

3. GLC separation of floridosides

GLC separation of floridoside from isofloridoside was achieved by using the pertrimethylsilylated derivatives. However, in spite of using a SE-30 fused silica capillary column and a variety of temperature programs, the D- and L-forms of isofloridoside could not be resolved one from the other (see

also Reed *et al.* 1980b, and Nagashima and Fukuda 1983). Separation of permethylated floridosides gave essentially similar results as the TMS derivatives. The retention times of permethylated derivatives were: floridoside 3.06 min; isofloridoside 3.27 min, at an isothermal temperature of 190°C. Peracetylation, in contrast, enabled the complete separation of the two isomers of isofloridoside. At 230°C, the relative retention times were mannitol (internal standard) 1.00 (1.88 min), D-form 3.07, and L-form 3.17. This is consistent with the reported separation of derivatised diastereoisomeric glycosides on a non-chiral phase capillary GLC column, which has been accomplished in the determination of D and L monosaccharides (trimethylsilylated (-)-2-butyl glycosides of D-, and L-arabinose, D- and L-rhamnose, D- and L-fucose, D- and L-ribose, D- and L-xylose, D- and L-mannose, D- and L-galactose, and D- and L-glucose, Gerwig *et al.* 1978; and acetylated (+)-2-octyl glycosides of D- and L-rhamnose, D- and L-fucose, D- and L-arabinose, D- and L-xylose, D- and L-mannose, D- and L-galactose, and D- and L-glucose, Leontein *et al.* 1978). Under the same conditions, floridoside has the same retention time as the L-form of isofloridoside. By using a temperature program from 100°C to 250°C at a rate of $2°C.min^{-1}$, floridoside can be partially separated from the L-form of isofloridoside.

Hence for routine analysis, a combination of acetylation and either trimethylsilylation or methylation can be used to quantify the floridoside and the two isomers of isofloridoside.

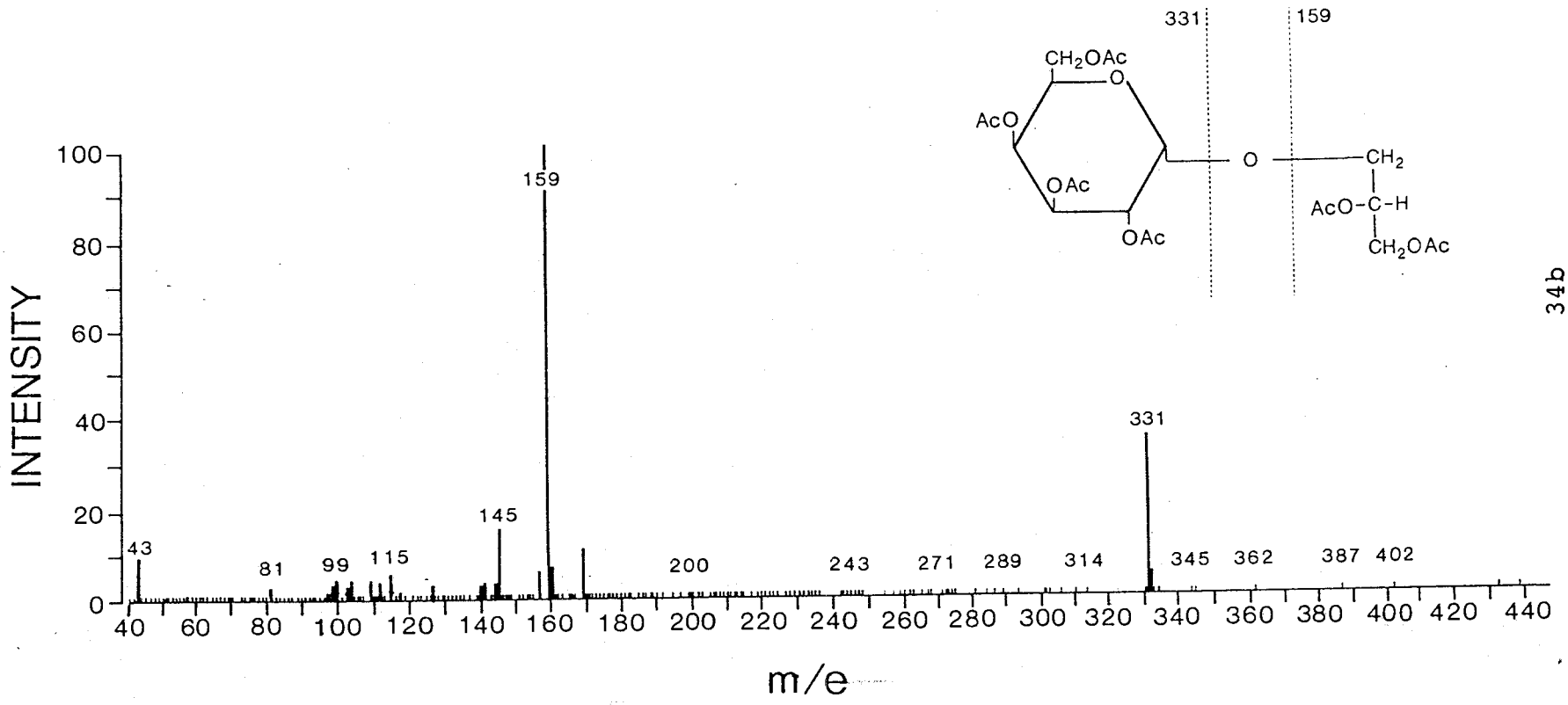
This method avoids the time-consuming separation of isofloridoside from floridoside by PC.

4. GC-MS analysis of floridosides

The mass spectrum of pertrimethylsilylated floridosides showed the following major fragments with their relative intensities in parentheses; floridoside m/e: 361 (8), 337 (26), 217 (20), 204 (100), 147(10), 103 (9); D- and L-isofloridoside m/e: 361 (28), 337 (18), 217 (26), 204 (100), 147 (15), 103 (13). No peak was observed at m/e > 491. DeJongh *et al.* (1969) reported that in the mass spectra of glycofuranosides, the relative intensity of the fragment at m/e 204 ($Me_3SiO\text{-}\overset{+}{C}H\text{-}\overset{\cdot}{C}\text{-}OSiMe_3$), which is one of the most intense peaks in the mass spectra of glycopyranoside, is drastically reduced. The reverse is true for the peak at m/e 217 ($Me_3SiO\text{-}CH{=}CH\text{-}\overset{+}{C}\text{-}HOSiMe_3$). Kamerling *et al.* (1971) confirmed this observation for most disaccharides. The intensity ratios of trimethylsilylated floridosides at m/e 204 and 217 being >>1, which indicate that the galactose moieties of these sugars are all in the pyranose forms.

GLC-MS analysis of permethylated floridoside and isofloridoside gave a similar fragmentation pattern, which is shown below; floridoside: m/e 278 $(M - CH_3\text{-}CH_2OCH_3)^+$, 219 (7), 187 (41), 163 (100), 155 (low intensity), 103 (14), 101 (35), 88 (62), 71 (13), and 45 (15); and D- and L-isofloridoside: m/e

Fig. 3. Mass spectrum of peracetylated L-form of isofloridoside.


INTENSITY
100
80
60
40
20
0
159
145
43
81
99
115
200
243
271
289
314
331
345
362
387
402
40 60 80 100 120 140 160 180 200 220 240 260 280 300 320 340 360 380 400 420 440
m/e
331
159
CH2OAc
O
AcO
OAc
OAc
O
CH2
AcO-C-H
CH2OAc

34b

278, 219 (low intensity), 187 (22), 163 (100), 103 (19), 101 (46), 88 (82), 75 (9), 71 (14), 45 (13). Lönngren and Svensson (1975) showed that for permethylated oligosaccharide alditols, their mass spectra give peaks corresponding to the fragment routes of permethylated glycosides and permethylated alditols. In addition, a fragment from C-1 of glycoside moiety linked to alditol ($MeO-C=\overset{+}{O}$-alditol moiety) is common. The most abundant peak m/e 163 corresponds to this fragment and derives from glycerol and part of galactose, which confirms the glycosidic linkage of galactose to glycerol. Hydrolysis of the permethylated isofloridosides and floridoside yielded 2,3,4,6-tetra-O-methyl-D-galactopyranose, as indicated by GLC-MS, which is also consistent with the structures.

The mass spectrum of the acetylated L-form of isofloridoside is presented in Fig. 3. The spectrum shows two major fragments at m/e 159 (100) and m/e 331 (35), which arise from the cleavage of the glycosidic linkage and correspond to the glycerol and galactose moieties, respectively. Most other fragments derive from the tetra-acetyl pyranoside ring. Floridoside and the D-form of isofloridoside have spectra similar to L-form of isofloridoside. The most obvious difference is the intensity at m/e 331, which is 75, 41 and 35%, respectively. Hydrolysis of the floridosides yielded D-galactose and glycerol in equal amounts as indicated by GLC-MS.

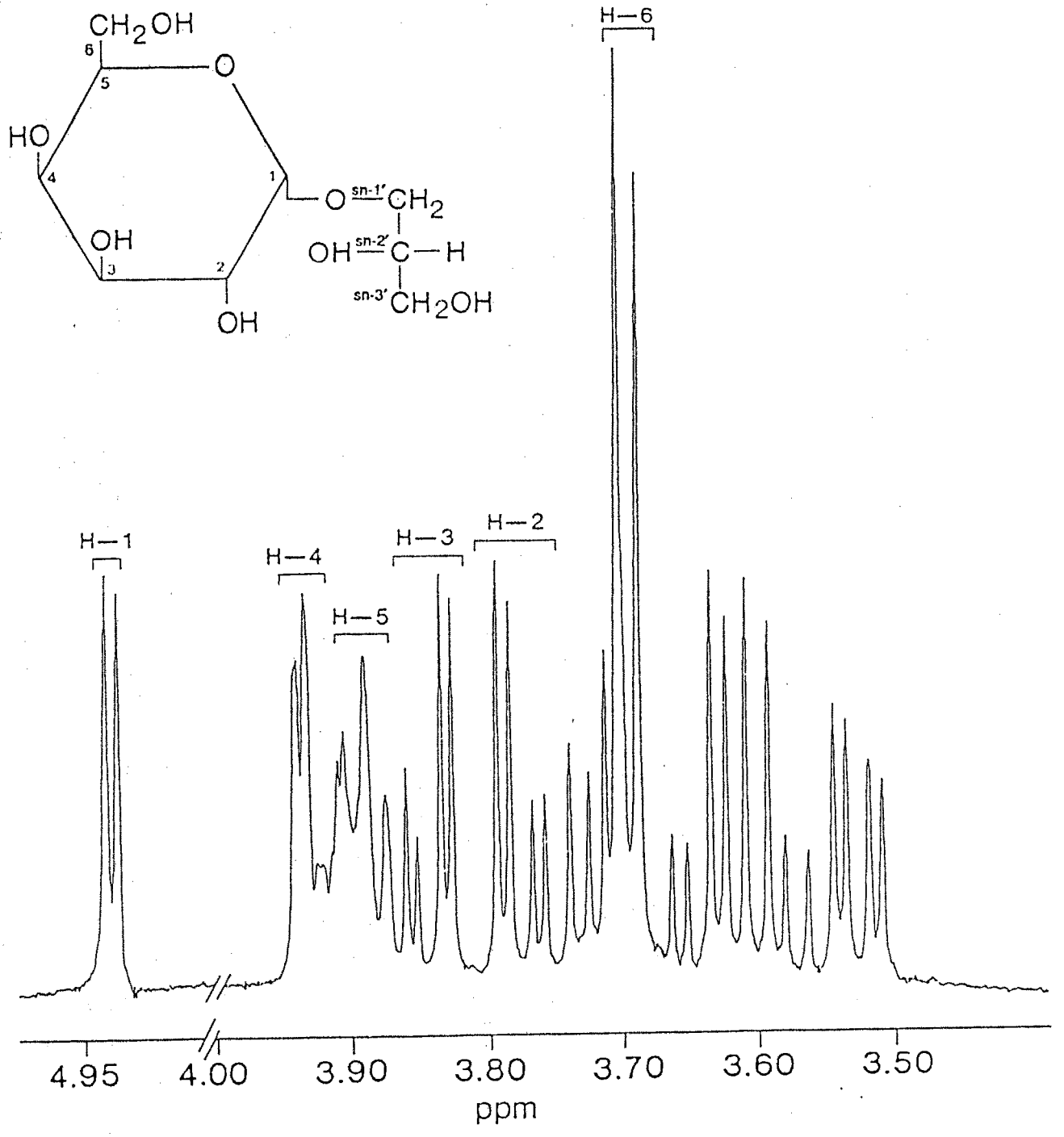
GLC-MS alone is inadequate in providing unambiguous information on the type of glycosidic linkage although

differences in intensity were observed for certain ion peaks, e.g. for acetylated derivatives at m/e 331, which may help to distinguish floridoside and isofloridoside. It is well known, however, that intensity can be different using different instruments; even using the same instrument at different times will give slightly different intensities due to slight changes in conditions, this can be seen by comparison of reported mass data for floridoside or isofloridoside (Alpin *et al.* 1967, Nagashima and Fukuda 1983, and Van Der Kaaden *et al.* 1984).

5. NMR analysis of floridosides

The 400-MHz ^{1}H-NMR spectrum of the purified L-form of isofloridoside is presented in Fig. 4. The galactose H-1 signals showed a doublet at δ=4.93 ppm ($J_{1,2}$=3.7 Hz) confirming an glycosidic linkage between the galactopyranose residue and glycerol. The H-2, H-3, H-4 and H-5 resonances of galactose have been assigned on the basis of selective spin decoupling experiments. The coupling constants of this compounds were: $J_{1,2}$=3.7 Hz, $J_{2,3}$=10.3 Hz and $J_{3,4}$=3.2 Hz. A slight overlap between the H-4 and H-5 signals rendered the determination of the coupling constant between these nuclei difficult and this problem was not pursued. Our ^{1}H-NMR spectrum of floridoside (data not shown) is similar to the reported 500-MHz ^{1}H-NMR spectrum (Lönngren and Svensson 1975) and the assignments of the protons, based on the decoupling experiment, are consistent with those published (Lönngren and Svensson 1975).

Fig. 4. 400-MHz ^{1}H-NMR spectrum of L-form of isofloridoside recorded in D_2O at 21°C. Chemical shift values are presented relative to internal acetone (δ=2.20 ppm).


CH2OH
6
5
O
HO
4
1
O sn-1' CH2
OH sn-2' C H
sn-3' CH2OH
OH
3
2
OH
H—6
H—1
H—4
H—5
H—3
H—2
4.95
4.00
3.90
3.80
3.70
3.60
3.50
ppm

The ^{1}H-NMR of isofloridoside (Fig. 5), isolated from a neutral fraction of *P. perforata* by PC, showed two isomers in almost equal amounts with two partially superimposed doublets at 4.93 and 4.94 ppm which are in the anomeric region. Since the L-form of isofloridoside only shows a doublet at 4.93 ppm the resonance at 4.94 ppm belongs to the D-form of isofloridoside. Furthermore, there is an additional multiplet in the area of 3.42-3.47 ppm. Since ^{1}H-NMR spectra of L-form of isofloridoside did not show any signal at this range, this multiplet corresponding to a single proton at the sn-2' position is characteristic of the D-form. These facts can be utilized for quantitative purpose. Since the chemical shift of the anomeric proton of floridoside is 5.14 ppm, which is well separated from those of the D- and L-forms of isofloridoside (4.93 and 4.94 ppm) all three floridosides can be quantitatively determined by ^{1}H-NMR.

The ^{13}C-NMR spectrum of the neutral fraction of *P. perforata* is shown in Fig. 6. The anomeric signals at 99.2, 99.6 and 99.9 ppm indicate the presence of three sugars. By comparison with standards, these three sugars were identified as floridoside (99.2 ppm), D-form of isofloridoside (99.9 ppm) and L-form of isofloridoside (99.6 ppm). The ^{13}C-NMR chemical shifts and the assignments of floridoside and D- and L-forms of isofloridoside are presented in Table 1. Comparison of these data with each other, as well as with those of model compounds, α-D-galactopyranose, methyl α-D-galactopyranoside, and glycerol,

Fig. 5. 400-MHz ^{1}H-NMR spectrum of isofloridoside (a mixture of D- and L-forms) recorded in D_2O at 21°C. Chemical shift values are presented relative to internal acetone (δ=2.20 ppm).

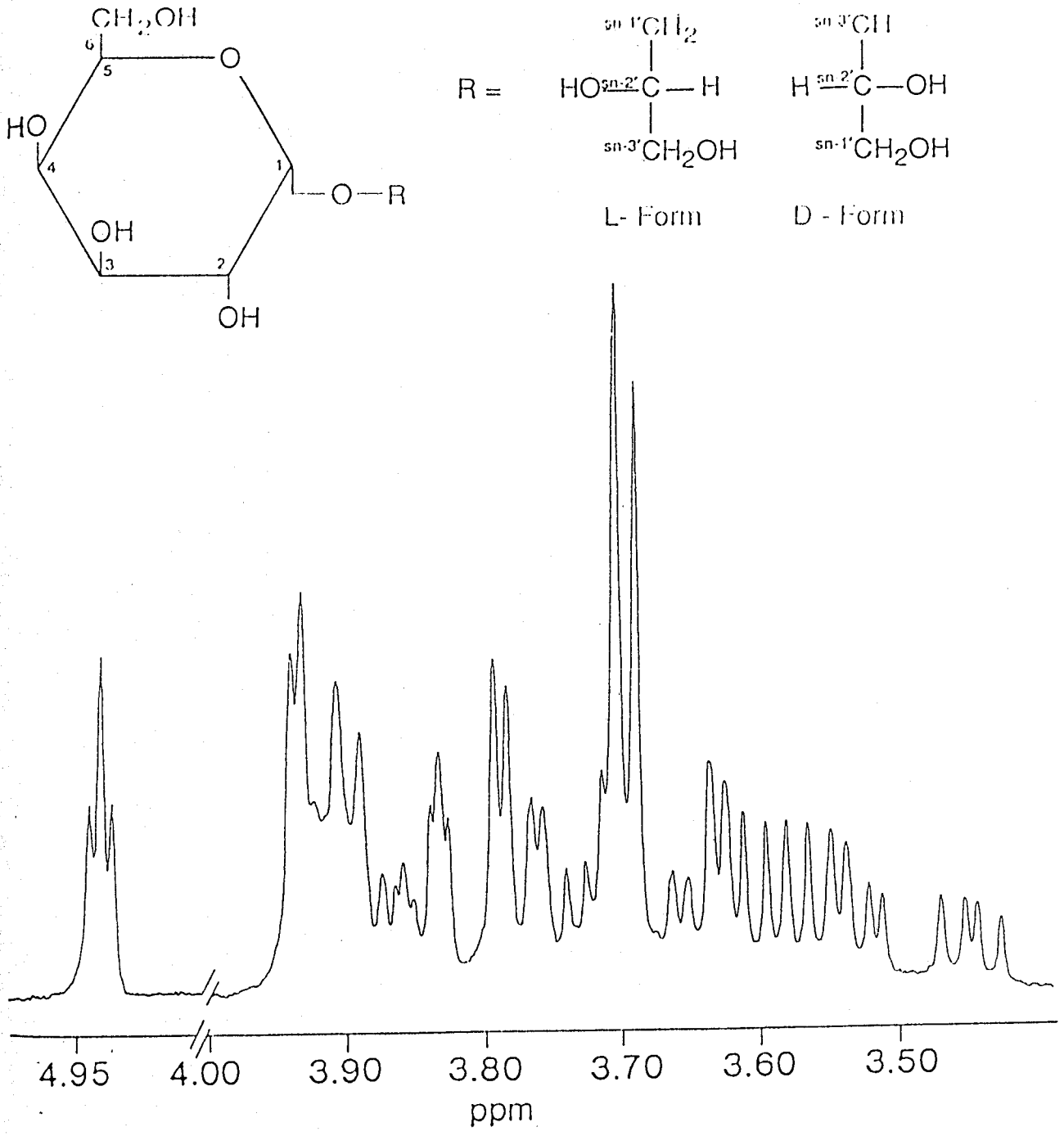
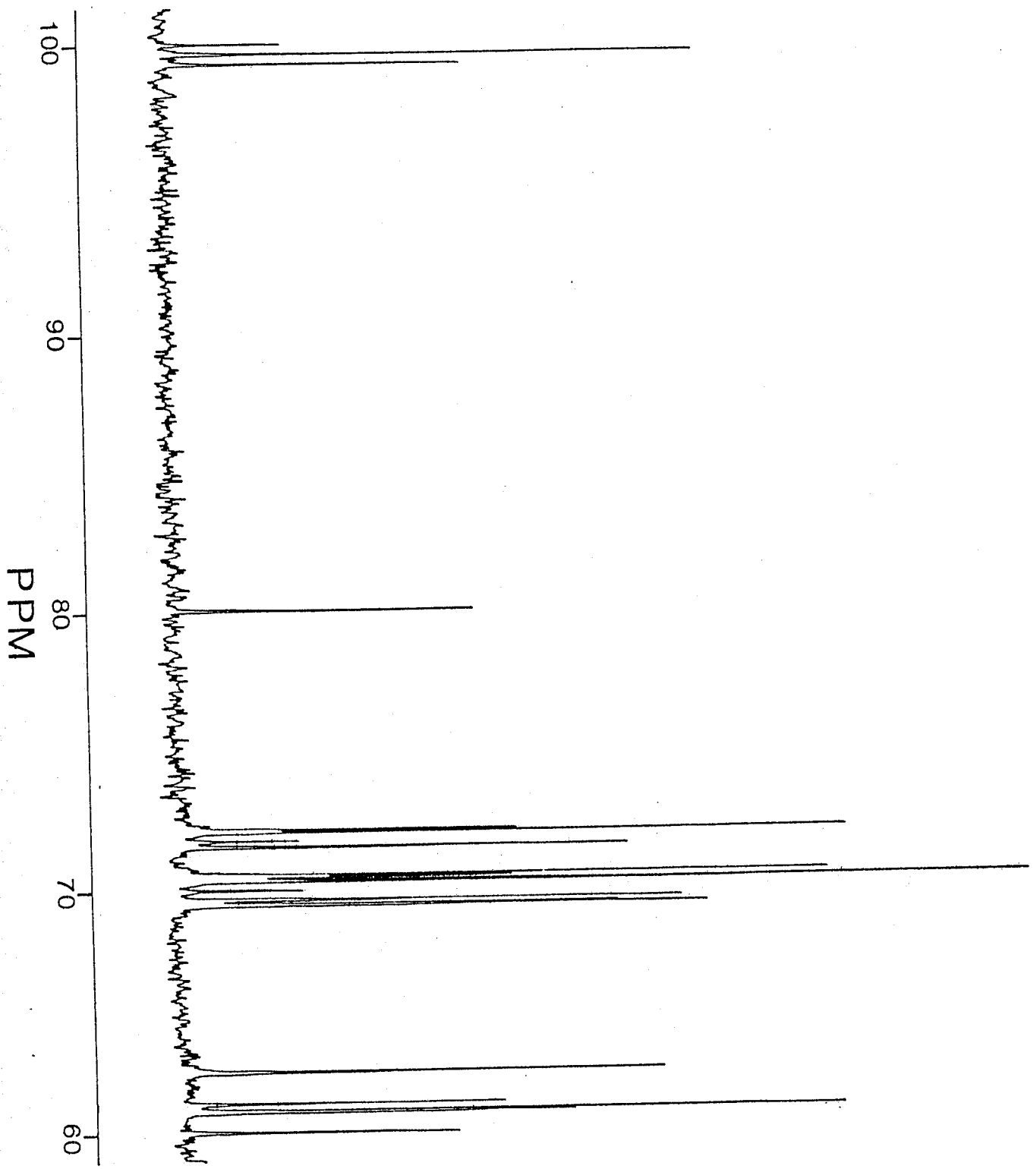

CH2OH
6
5
O
HO
4
1
O—R
OH
3
2
OH
R =
sn-1'CH2
HO sn-2' C — H
sn-3'CH2OH
sn-3'CH
H sn-2' C — OH
sn-1'CH2OH
L- Form
D - Form
4.95
4.00
3.90
3.80
3.70
3.60
3.50
ppm

Fig. 6. 400-MHz ^{13}C-NMR spectrum of floridoside and isofloridoside (D- and L-forms) recorded in D_2O at 21°C. Chemical shift values are relative to internal acetone (δ=31.4 ppm).


PPM
100
90
80
70
60

makes the complete assignments possible. The α configuration of the D-galactose residues in the three floridosides is evident from the resonances of the anomeric carbons in the region of 99-100 ppm, which was statistically established as the α configuration region for galactopyranosides (Beier *et al.* 1980). ^{13}C-NMR chemical shifts of floridoside and D- and L-forms of isofloridoside showed that most carbons in D-galactopyranose residues, except the anomeric carbons, had almost identical chemical shift values except for a minor difference in the C-5 signal (see Table 1), which resonated 0.7-0.8 ppm downfield compared to the C-5 signal of α-D-galactopyranose. This displacement can be attributed to the nature of the aglycone as no similar displacement occurs in the C-5 signal of methyl α-D-galactopyranoside. The biggest difference between D- and L-forms of isofloridoside is the 0.3 ppm downfield shift for the sn-2' signal of the D-form. The fact that in floridoside three free hydroxymethyl signals (at 61.5, 62.3 and 62.5 ppm) appear in the spectrum indicates that both hydroxymethyl groups of the glycerol moiety must be unsubstituted and primary, implying that the linkage occurs at sn-2' (79.9 ppm). This is in contrast to the D- and L-forms of isofloridoside, which show only two free hydroxymethyl signals (at 62.3 and 63.7 ppm, respectively), suggesting the linkage occurs at sn-1' and sn-3'. Compared to glycerol, the large downfield shifts at sn-2' for floridoside (6.6 ppm) and at sn-3' for D- for and sn-1' for L-forms of isofloridoside (8.0 and 7.8 ppm) also support these assumptions.

Table 1 Carbon-13 Chemical Shifts of Floridosides and Other Relevant Compounds

Compounds[a]	Chemica shifts[b c] in p.p.m.								
	Galactosyl group						Glycerol residue		
	C-1	C-2	C-3	C-4	C-5	C-6	C--1′	C-2′	C-3′
Floridoside	99.2	69.6	70.5	70.4	72.2	62.3	62.5	79.9	61.5
Isofloridoside (D-form)	99.9	69.6	70.6	70.4	72.1	62.3	63.7	70.1	71.8
Isofloridoside (L-form)	99.6	69.8	70.6	70.4	72.1	62.3	71.6	69.8	63.7
Methyl α-D-Galp[d]	100.1	69.2	70.5	70.2	71.6	62.2			
α-D-Galactopyranose[d]	93.2	69.4	70.2	70.3	71.4	62.2			
Glycerol[e]							63.8	73.3	63.8

a. Galp = galactopyranosyl or galactopyranoside
b. Assignments for C-3 and C-4 of floridoside, D-isofloridoside and L-isofloridoside may be reversed.
c. Assignments for C-6, C′-1, and C′-3 of floridoside; C-6 and C′-3 of L-isofloridoside; and C-6 and C′-1 of D-isofloridoside may be interchanged.
d. Taken form Bock and Thogersen (1982).
e. Taken from De Boer *et al.* (1976).

DISCUSSION

^{13}C-NMR proved to be the most powerful method in the present analyses. Most carbon atoms in the three isomers could easily be distinguished. For floridoside, our assignment coincides with that of Van Der Kaaden *et al.* (1984). There is a 0.1 ppm difference between our D-form of isofloridoside chemical shift data and those of Beier et al (1980), except at the C-5 position where the shift difference was 0.5 ppm. Since they did not publish the whole assignment of isofloridoside and did not distinguish its D- and L- forms, it is difficult to compare their data with the data reported here. Their C-3 and C-4 signals were not resolved, but a 0.2 ppm difference was observed. It is also possible that there are some temperature-related variations which may cause the above differences.

In preliminary experiments, we have noted that the ratios of D- and L-forms of isofloridoside in *P. perforata* change from time to time, e.g. the sample collected at Stanley Park, Vancouver, on Nov. 20, 1984, had a D- to L-ratio of almost 1; while for a sample collected at the same place on May 20, 1985, the ratio was 0.01. Thus the contradictory reports(Craigie *et al.* 1968, Wickberg 1958, Peat and Rees 1961, and Su and Hassid 1962) about isofloridoside forms could be explained by different proportions of D- and L-forms in the analyzed materials collected at different times and locations.

Isofloridoside was found in larger amount than floridoside in *P. perforata*. Many studies have dealt with the possible physiological function of floridosides, but they did not distinguish between D- and L-forms of isofloridoside, and sometimes not even between floridoside and isofloridoside. Methods presented here can be used to analyze each isomer of the floridosides for future study.

CHAPTER 4

EXTRACTION, ASSAY AND SOME PROPERTIES OF FLORIDOSIDE PHOSPHATE SYNTHASE FROM *PORPHYRA PERFORATA*

MATERIALS AND METHODS

1. Materials

Porphyra perforata J. Ag. collected from Stanley Park, Vancouver, was cleaned of visible epiphytes and epifauna. It was washed with 0.2 μm membrane filtered seawater, squeezed of excess water by a nylon net, frozen immediately in LN_2, and stored at -80°C. Under these storage conditions, no loss of extractable FPS activity was observed for at least 6 months.

2. Extraction

All extraction steps were carried out at 4°C, unless otherwise specified. Fifteen g material was frozen in liquid nitrogen and ground to a fine powder, which was extracted with 40 ml 50 mM Tricine buffer (pH 8.0), containing 5 mM $(NH_4)_2SO_4$, 2 mM ethylenediamine tetraacetate (EDTA), 5 mM dithiothreitol (DTT), and 10% polyvinylpolypyrrolidone (PVPP, w/v) for 10 min. PVPP was pretreated according to Loomis (1974) and equilibrated in extraction buffer overnight before use. The extract was passed through 6 layers of cheese cloth and centrifuged at 5,000 X g for 10 min. The supernatant was desalted on a G-25 gel filtration column (2.5 X 20 cm) using 50 mM Tricine buffer containing 5 mM $(NH_4)_2SO_4$. Protein fractions detected by UV monitoring at 280 nm were collected and pooled, designated as crude extract, and assayed. The whole process was done within 1

h. To prepare concentrated protein solutions, ammonium sulfate was added to the crude extract to 80% saturation, equilibrated for 1 h and centrifuged at 20,000 X g for 30 min. The pellet collected was dissolved in 4 ml Tricine buffer (50 mM, pH 8.0), equilibrated at room temperature overnight, and centrifuged at 12,000 X g for 5 min to clarify the protein solution.

3. Protein measurement

Protein concentrations were determined by the dye-binding method of Bradford (1976). Bovine serum albumin (BSA) was used as a standard.

4. Carbohydrate measurement

Total carbohydrate in protein solutions was determined by the phenol-sulfuric acid method (Kochert 1978), galactose was used as a standard.

5. Phenolic compound measurement

Total phenol content in protein extracts was estimated by the Folin-Denis colorimetric method (Swain and Hillis 1959, Horwitz 1970) as suggested by Ragan and Craigie (1978), with phenol serving as the standard.

6. Assay of FPS activity

The assay was based on the measurement of the formation of one of the products, floridoside phosphate or UDP. The incubation solution consisted of 50 μl buffer (50 mM Tricine, pH 8.0), 0.5 M $(NH_4)_2SO_4$ 10 mM UDP-GAL, 40 mM GLYP, and 5 mM Mg^{2+} The incubation was at 30°C for 15 min and the amount of protein was adjusted so that the product formed in 15 min was less than 10% of the substrates added at the beginning of the reaction. The reaction was started with the addition of 30 μl of the crude extract. For GLC assay, 20 μg mannitol was added to the reaction mixture as an internal standard at the end of the reaction. The reaction was stopped by heating the incubation sample at 100°C for 1 min. Samples were centrifuged at 12,000 X g for 5 min, and the supernatant was used for product analysis.

Assay methods

Assay method 1: measurement of floridoside by gas-liquid chromatography (GLC).

The product from the FPS catalyzed reaction, floridoside phosphate, was dephosphorylated by one of two methods:

1. Hydrofluoric acid method: The incubation mixture was freeze-dried, 1 ml of 48 % HF was added to the sample and kept at 4°C for 4 days. Then Dowex 1 (OH^-) was added to adjust the pH of the solution to 7. The sample was further purified by Dowex 1

(OH^-) and Dowex 50 (H^+) ion exchange resins using batch method.

2: Phosphatase method: Acid phosphatase (Sigma, type II from potato) was used to cleave the phosphate group from floridoside. The incubation mixture was diluted to 0.4 ml with distilled water, and the pH was adjusted to pH 5 using 0.25 N HCl. 2 mg (1.6 unit) acid phosphatase was added to the solution and incubated at 37°C for 2 h. The reaction was stopped by adding 1 ml absolute ethanol and protein was precipitated out by centrifugation at 12,000 X g for 10 min.

GLC analysis of the product: Each sample was vacuum evaporated to dryness below 40°C, trimethylsilylated by TRI SIL 'Z' (Pierce; Rockford, IL) at 60°C for 30 min, and injected directly into GLC. GLC was performed in a Hewlett-Packard 5790A instrument equipped with a flame-ionization detector and connected to a model 3390A electronic integrator. A fused-silica capillary column (SE-30, 30 m X 0.2 mm) was employed, with helium as the carrier gas. The presence of floridoside was confirmed by combined gas-liquid chromatography-mass spectrometry (GLC-MS). GLC-MS was carried out on a Hewlett-Packard 5985B GC/MS/DS using the above column and an ionization potential of 70 eV.

The total amount of floridoside was calculated using mannitol as an internal standard with the response factor calibrated against 20 μg mannitol and different concentrations of pure floridoside at the same conditions.

Assay method 2: Measurement of GLYP dependent formation of UDP. To measure the UDP formed in the reaction (Salerno and Pontis, 1978), each tube was supplemented with 100 µl solution containing 150 mM Tricine (pH 8.0), 4 mM phosphoenolpyruvate, 150 mM KCl, 10 mM $MgCl_2$, and 20 units ml^{-1} of pyruvate kinase (Type III, from rabbit muscle; Sigma). After incubation at 37°C for 15 min, each tube received 150 µl of 0.1% 2,4-dinitrophenylhydrazine in 2 M HCl. After 5 min at room temperature, 0.2 ml of 10 N NaOH and 0.5 ml ethanol were added. The tubes were mixed and after centrifugation at 12,000 X g for 3 min, the A_{520} was measured for the amount of pyruvic 2,4-dinitrophenylhydrazone formed.

Improvement of the assay procedure

In an effort to improve the enzyme assay procedure, different incubation conditions were tested for enzyme assay, including incubation time, temperature, different concentrations of protein.

All experiments reported here were repeated at least three times and each sample was measured in triplets.

RESULTS

1. Enzyme assays

(1). Dephosphorylation of floridoside phosphate

It is not clear yet which enzyme catalyzes dephosphorylation of floridoside phosphate *in vivo*. In our experiments, no free floridoside was detectable in the assay mixture.

Dephosphorylation by aqueous hydrofluoric acid (HF) has been used for many carbohydrate phosphate esters with a recovery rate of ca. 94% (Lipkin *et al.* 1969); the hydrolysis of glycosidic bonds is considered insignificant (Prehm *et al.* 1975). This method has also been used for polymerized floridoside phosphate (Van Der Kaaden et al. 1984). Dephosphorylation by acid phosphatase has been reported for other carbohydrate phosphates (Ohashi *et al.* 1984). Both methods were used in this part of the study. When using the same amount of floridoside phosphate, the recovery rates of these two methods were comparable; the discrepancy in product yields was less than 4% (Table 2). However, the HF method was found to be tedious and time consuming and was not used in later work. In this assay, floridoside rather than floridoside phosphate was used for measuring enzyme activity, this is because 1. methods for floridoside detection and quantitation are well established; 2. purification of floridoside phosphate from the assay mixture, which includes a number of compounds containing phosphate esters

is difficult and this was not pursued.

(2). Product analysis

The trimethylsilylation method was chosen for GLC analysis because of good separation between floridoside and isofloridoside (Meng *et al.* 1987; see also Section 3). The presence of floridoside in the sample was frequently checked by GLC-MS. UDP assay provides the an alternative assay method. The results provided by this assay are comparable to those from GLC analysis (Table 2) over a range of substrate concentrations (Table 3). Since UDP-GAL could be the precursor for other reactions to form polysaccharides, or could be broken down nonspecifically, this method can also be used to estimate the GLYP-independent metabolism of UDP-GAL (Table 4). It was found that the GLYP-independent formation of UDP was insignificant. In the UDP assay method, protein extract gave a background absorbance at A_{520}, probably due to pigments from phycobiliproteins. For the same batch of protein preparation, therefore, protein concentrations were kept the same among different samples to correct for this background absorbance.

2. Extraction method

FPS activity was compared after different extractions in which one or another component of the extraction buffer was omitted or added (Table 5). Unlike the case of brown seaweeds (Marsden *et al.* 1981), ascorbic acid did not enhance the enzyme

Table 2. Comparison of Two Assay Methods for Measurement of Floridoside Phosphate Synthase Activity

Assay Method	Protein in Reaction (mg)	Product Formed[a]	
		Floridoside	UDP
GLC Assay			
HF Method	0.1	0.326 ± 0.031	--
Phosphatase Method	0.1	0.346 ± 0.007	--
UDP Assay	0.1	-	0.377 ± 0.015

a. The unit is μmol h^{-1} mg^{-1} protein.

Table 3. Comparison of GLC and UDP assay methods under different concentrations of UDP-GAL.

UDP-GAL Concentrations	Product Formed[a]	
	GLC Method	UDP Method
1.0 mM	0.62 ± 0.03	0.61 ± 0.03
2.5 mM	1.02 ± 0.11	1.03 ± 0.02
5.0 mM	1.39 ± 0.08	1.39 ± 0.03
7.5 mM	1.54 ± 0.06	1.56 ± 0.02
10.0 mM	1.59 ± 0.09	1.49 ± 0.02

a. The unit is μmol h^{-1} mg^{-1} protein.

Table 4. Metabolism of UDP-galactose

Sample	Protein in Reaction (mg)	Absorbance A_{520}	UDP Formed[a]
Protein + UDP-GAL[b] (without incubation)	0.1	0	0
Protein + UDP-GAL[c]	0.1	0	0
Protein + UDP-GAL[d] +GLYP	0.1	0.377 ± 0.015	3.8

a. The unit is μmol h^{-1} mg^{-1} protein.
b. The reference sample.
c. GLYP-independent production of UDP.
d. GLYP-dependent production of UDP.

Table 5. Effect of Different Components in Extraction Buffer on the activity of the FPS

Extraction Buffer	FPS Activity[a] (μmol h^{-1} g^{-1}fresh wt)	Percent Change
Complete tricine buffer[b]	6.9	0
Tricine buffer plus ascorbic acid	6.3	-10
Tricine buffer plus Triton X-100	5.7	-21
Tricine buffer plus Tween 80	7.4	+7
Tricine buffer minus PVPP	7.3	+6
Tricine buffer minus DTT	4.7	-32
Tricine buffer plus BSA	9.6	+41

a. Assayed in assay buffer (see materials and methods).
b. Complete tricine buffer is extraction buffer (see Materials and Methods).

activity of the crude extract. It was not used in later extractions. Addition of Triton X-100 to extraction buffer decreased, whereas the addition of 0.2% (v/v) Tween 80 slightly increased the extractable enzyme activity (Table 5). Unfortunately, protein preparations with detergent had a tendency to float on the surface (probably lipid and lipid-proteins, see Rice and Crowden 1987, for brown algae) during ammonium sulfate precipitation to concentrate protein, and hence detergents were not used in most extractions. Most phenols were low molecular weight compounds the majority of which were removed by a Sephadex G-25 gel filtration step (Table 6). Even though PVPP did not help the extractable enzyme activity, it was included in the extraction buffer to absorb any phenolic compounds present in the extract. The reason why PVPP did not have a greater effect in enhancing the enzyme activity could be that the pH value (8.0) used in extraction limited the effectivness of PVPP. An ion-exchange column was one of the most effective ways of removing polysaccharides from the protein solution. Less than 10% of the original polysaccharides were left after the ion-exchange column (Table 6). BSA (1%, w/v) enhanced the extractable enzyme activity probably by preventing enzyme oxidation and absorbing some harmful compounds; it was included in extraction buffer when estimating the total FPS activity in fresh tissue. But if further purification of the enzyme or electrophoresis was desired, BSA interfered with these processes and was omitted.

Table 6. Content of phenolic compounds and carbohydrates in protein fractions.

Sample	Phenolic Compounds (mg g^{-1} f wt)	Carbohydrates (mg g^{-1} f wt)
5,000 X g Supernatant	30.44	–
Sephadex G-25	3.48	22.95
DEAE Ion-Exchange	2.23	4.46
Ammonium Sulfate pptd protein	0.32	0.98

FPS appears to be a soluble enzyme because a substantial amount of enzyme activity can be detected in the supernatant after centrifugation at 100,000 X g (Table 7). Under these conditions, membranes and other subcellular components are centrifuged out in the pellets and there was no significant FPS activity in the pellets (Table 7). Also, as stated earlier, addition of detergent Tween 80 only slightly increased the extracted FPS activity.

In various experiments using optimal extraction and assay conditions, the FPS activity ranged from 4-7 μmol product formed $h^{-1}.g^{-1}$ fresh wt. Majak *et al.* (1966) studied the photosynthetic rate and carbon partitioning of photosynthetic products in *Porphyra umbilicalis*. They found that *P. umbilicalis* had a photosynthetic rate of 90 μmol C h^{-1} g^{-1} fresh wt, and ca. 55% of total fixed carbon accumulated in floridoside. Considering 9 carbon atoms in floridoside, the floridoside biosynthetic rate will be 5.5 μmol product formed h^{-1} g^{-1} fresh wt. This figure agrees well with our extracted FPS activity. It may also indicate that this enzyme could be a rate-limiting step in the biosynthesis of floridoside.

3. Some properties of floridoside phosphate synthase

Table 7. FPS Activity in Supernatant and Pellet under Different Relative Centrifugal Force

Relative Centrifugal Force (X g)	Time (min)	FPS Activity (μmol h^{-1} g^{-1} fresh wt)
5,000 X g Supernatant	10	2.6
Pellet		1.1
20,000 X g Supernatant	30	2.8
Pellet		0.4
100,000 X g Supernatant	30	2.6
Pellet		0.2

(1). Temperature and protein concentration dependence of FPS activity

FPS activity was temperature dependent(Fig. 7). The activity gradually increased with temperature, and peaked at 35 °C. At 40 °C, the product, if formed, was below detection level, probably due to the denaturation of FPS. Considering the temperature profile, 30°C was chosen for subsequent assays.

FPS activity was linear with protein amounts from 0.04 to 0.17 mg per sample, those protein amounts are equivalent to protein concentrations of 0.8 to 3.3 mg ml^{-1} (Fig. 8).

(2). Stability of the enzyme

1. Enzyme stability at different temperatures in concentrated solutions.

Since there was some evidence that FPS was unstable (Kremer and Kirst 1981), several tests were done to check the stability of this enzyme. It was found that at -20°C, the 80% ammonium sulfate concentrated fraction (4 mg protein ml^{-1}) was stable for at least 1 month with only a slight decrease in activity (Table 8). At room temperature, the enzyme activity was stable over at least 5 days; whereas at 100°C, the enzyme activity was totally lost within 1 min.

2. Enzyme stability after dilution

Fig. 7. FPS activity assayed at different temperatures

(The temperature of the protein solution was adjusted to the desired value before the assay)

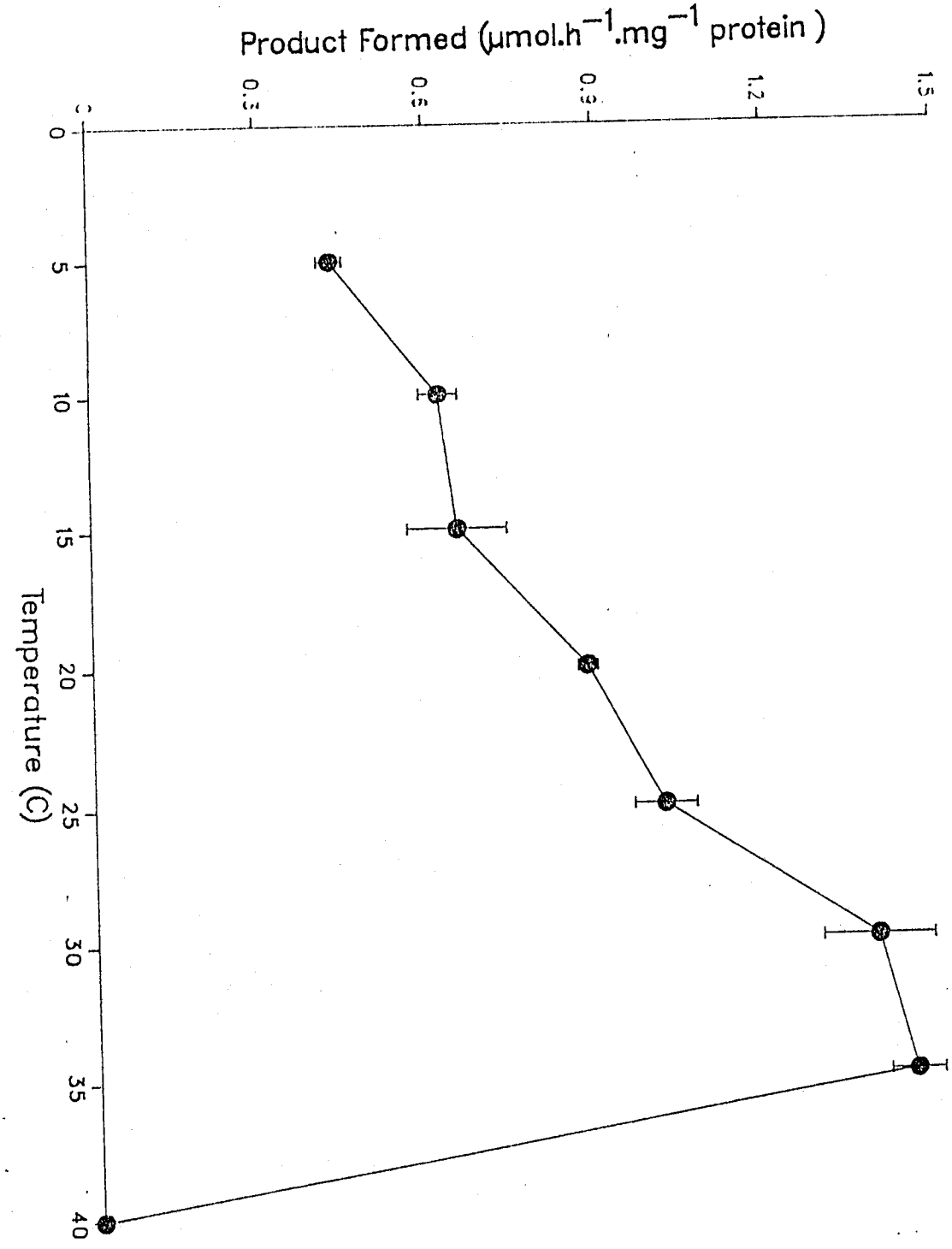

Product Formed (µmol.h^{-1}.mg^{-1} protein)
0.3
0.6
0.9
1.2
1.5
0
5
10
15
20
25
30
35
40
Temperature (C)

Fig. 8. FPS activity assayed at different protein concentrations

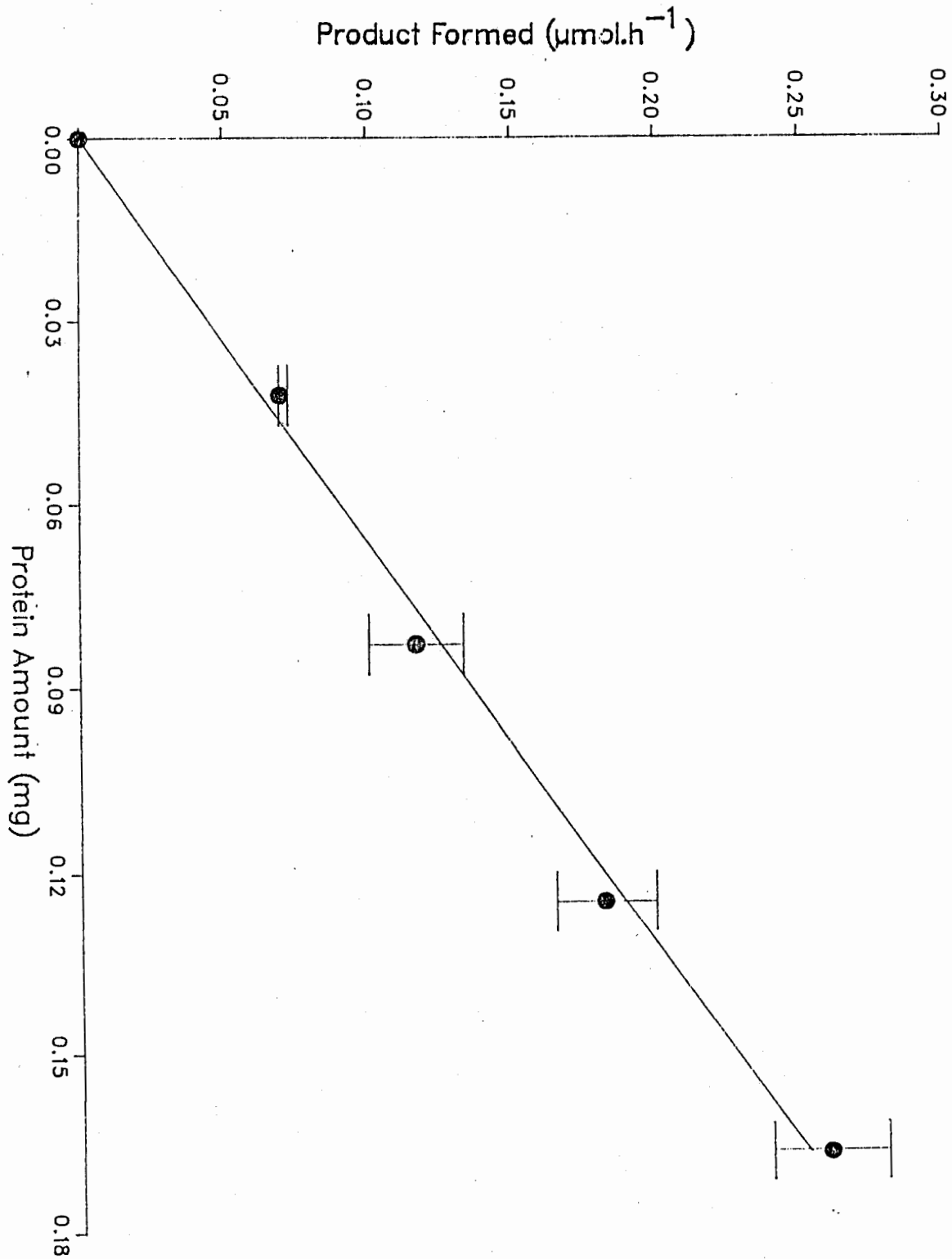

Product Formed (µmol.h^{-1})
0.05
0.10
0.15
0.20
0.25
0.30
0.00
0.03
0.06
0.09
0.12
0.15
0.18
Protein Amount (mg)

Table. 8. FPS Activity in Relation to Temperature and Duration

	Duration			
Temperature	1 min	1 day	5 days	30 days
-20°C	100%[a]	90.4%	88.2%	89.6%
22°C	100%	100%	97.8%	-[b]
100°C	0%	-	-	-

a. The activities are presented as percentage of enzyme activity at 0 time.
b. - indicates untested.

It was found that the enzyme immediately lost its activity in diluted solution (below 0.2 mg protein ml^{-1}). The loss of activity was too great to be accounted for by absorption on glass walls or protease degradation. The addition of 0.5 M ammonium sulphate, 10 mg ml^{-1} BSA, or 10% polyethylene glycol did not prevent the loss of activity. This phenomenon may be accounted for by assuming that the enzyme is an oligomer; it dissociates during dilution causing the loss of activity. Attempts to recover the lost activity by reconcentrating the protein solution with ammonium sulfate or Amicon membrane were unsuccessful.

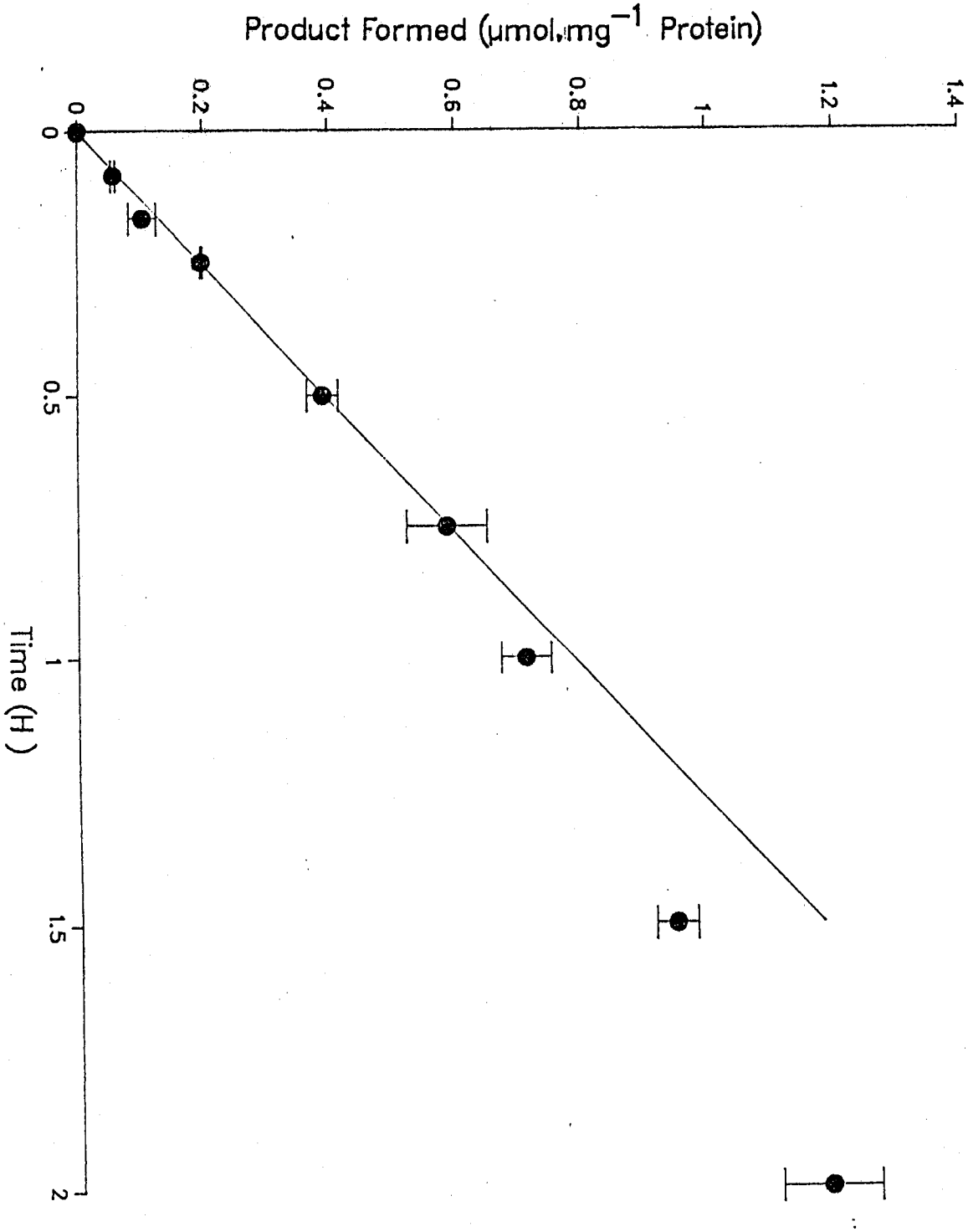
(3). Time course of FPS catalyzed reaction

Incubations for different lengths of time were tried. The formation of floridoside phosphate was linear for at least 45 min (Fig. 9). The falling off of activity beyond 45 min was attributed to the inhibitory effect of UDP formed from the reaction (see section 6) because no effect of instability of any substrate or enzyme was detected by preincubation of protein with each substrate. The effect of floridoside phosphate accumulation on FPS activity was not tested, because floridoside phosphate was unavailable.

(4). Substrate specificity of FPS

The specificity of this enzyme is an interesting problem. In red algae, isofloridoside is recorded only in species belonging to the order Bangiales. Such a restricted occurrence of

Fig. 9. The time course of FPS catalyzed reaction.


Product Formed (µmol.mg^{-1} Protein)
0
0.2
0.4
0.6
0.8
1
1.2
1.4
Time (H)
0
0.5
1
1.5
2

isofloridoside raises the possibility that its biosynthesis is due to a low substrate or regio specificity of FPS in the order Bangiales (Shibuya 1960). To test this possibility several possible combinations of substrates were tested and product checked for existence of isofloridoside. The results are shown in Table 9.

It is quite clear that this enzyme has a very high substrate and regio specificity. For all the substrates tested under identical conditions of incubation, not even a trace amount of isofloridoside could be detected. These data suggest that the biosynthesis of isofloridoside probably occurs via a different pathway.

The enzyme specificity for stereoisomers of glycerol phosphate was also studied. No product could be detected when sn-2-glycerol phosphate was used as a substrate. Since no sn-1-glycerol phosphate was commercially available, sn-3-glycerol phosphate was compared with sn-1, sn-3-glycerol phosphate mixture (rac-GLYP, assuming sn-1, sn-3- isomers were in a 1:1 ratio) at both saturating (10 mM UDP-GAL, 40 mM GLYP or 80 mM rac-GLYP) and limiting conditions (3 mM UDP-GAL, 10 mM GLYP or 20 mM rac-GLYP). Tests with the 2 stereoisomers of glycerol phosphate gave almost the same amounts of product under limiting conditions, which suggests that sn-1-glycerol phosphate is not used as a substrate for FPS. Under saturating conditions, GLYP formed more product than rac-GLYP mixture, which indicated that at high concentration, sn-1-glycerol phosphate inhibited

Table 9.
Substrate Specificity of FPS

Substrates	Product formed[a]	
	Floridoside phosphate	Isofloridoside phosphate
UDP-GAL[b] + GLY-2-p[c]	-[d]	-
UDP-GAL + Glycerol	-	-
GAL-6-p + rac-GLYP[e]	-	-
GAL-1-p + rac-GLYP	-	-
UDP-GAL + rac-GLYP (saturating concentration)[f]	0.68 ± 0.16	-
UDP-GAL + GLYP (saturating concentrations)	0.84 ± 0.06	-
UDP-GAL + rac-GLYP (limiting concentrations)[g]	0.60 ± 0.09	-
UDP-GAL + GLYP (limiting concentrations)	0.65 ± 0.05	-

a. The unit is μmol h^{-1} mg^{-1} protein.
b. GAL=galactose.
c. GLY-2-P=sn-2-glycerol phosphate.
d. rac-GLYP=an equal mixture of sn-1-, and sn-3-glycerol phosphate.
e. - indicates no product formed.
f. Saturating concentration: 10 mM UDP-GAL, 40 mM GLYP or 40 mM rac-GLYP.
g. Limiting concentration: 3 mM UDP-GAL, 10 mM GLYP or 20 mM rac-GLYP.

the FPS catalyzed reaction. Since no sn-1-glycerol phosphate occurs *in vivo*, the inhibition effect of sn-1-glycerol phosphate was not pursued.

(5). pH optimum of FPS

Enzyme activity measured in two buffers (50 mM Tricine pK 8.1 and 50 mM Bes pK 7.1) at different pH values is shown in Fig. 10. There was not much variation as to product formed among the pH values 6.5 to 8.5, but at pH 6.0 and pH 9.0 the enzyme activity was considerably reduced.

(6). FPS activity as affected by salts

FPS activity was found to be salt dependent. In the presence of ammonium sulfate, FPS activity increased initially with increasing concentration of $(NH_4)_2SO_4$, until ammonium sulfate concentration reached 0.1 M. It stayed at the same level up to an $(NH_4)_2SO_4$ concentration of 0.35 M and declined above a concentration of 0.5 M (Fig. 11). Some other salts tested showed similar effects on FPS activity under comparable concentrations (Table 10).

Fig. 10. FPS activity assayed at different pH values.

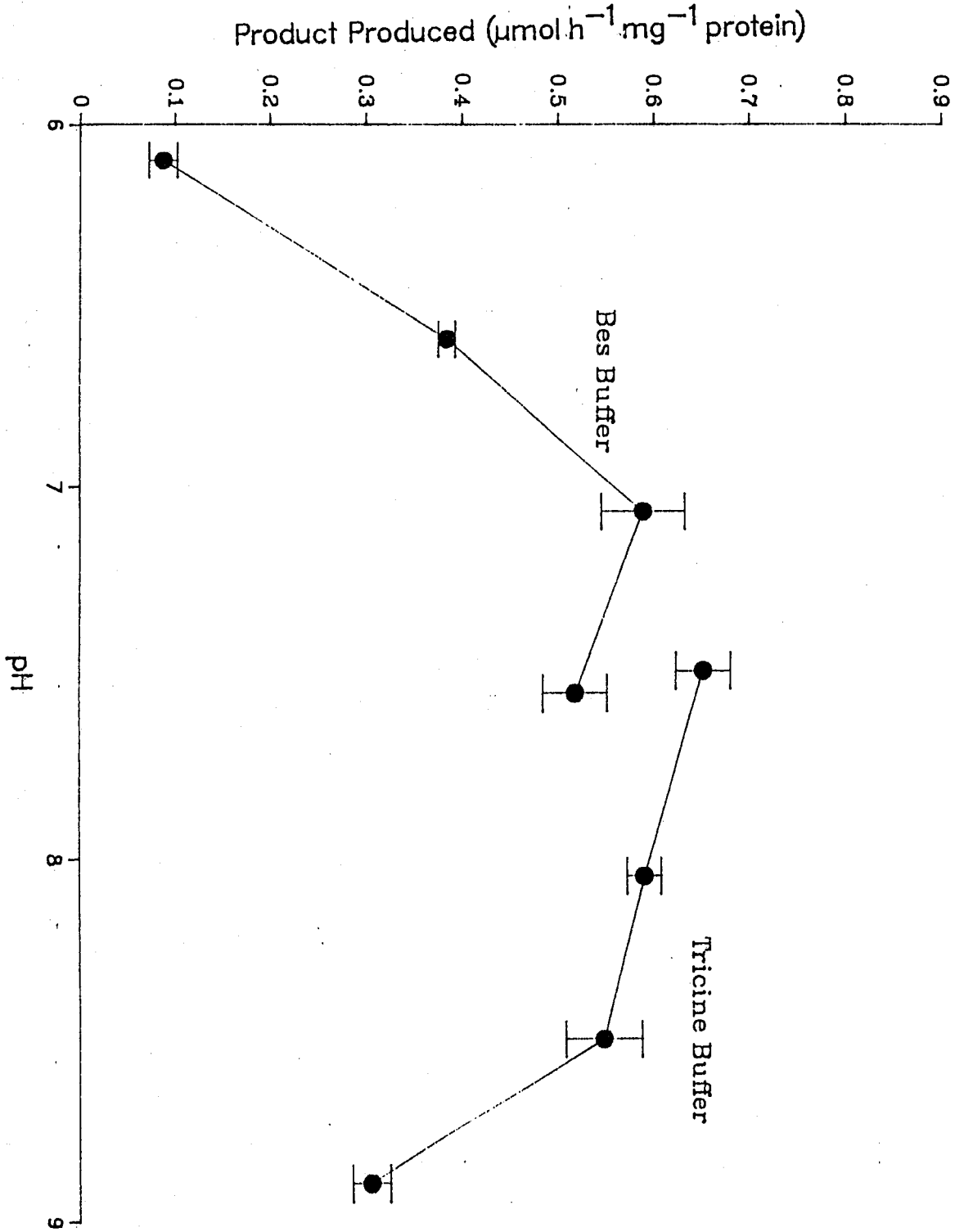

Product Produced (μmol h⁻¹ mg⁻¹ protein)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
6
7
8
9
pH
Bes Buffer
Tricine Buffer

Fig. 11. FPS activity assayed at different ammonium sulfate concentrations

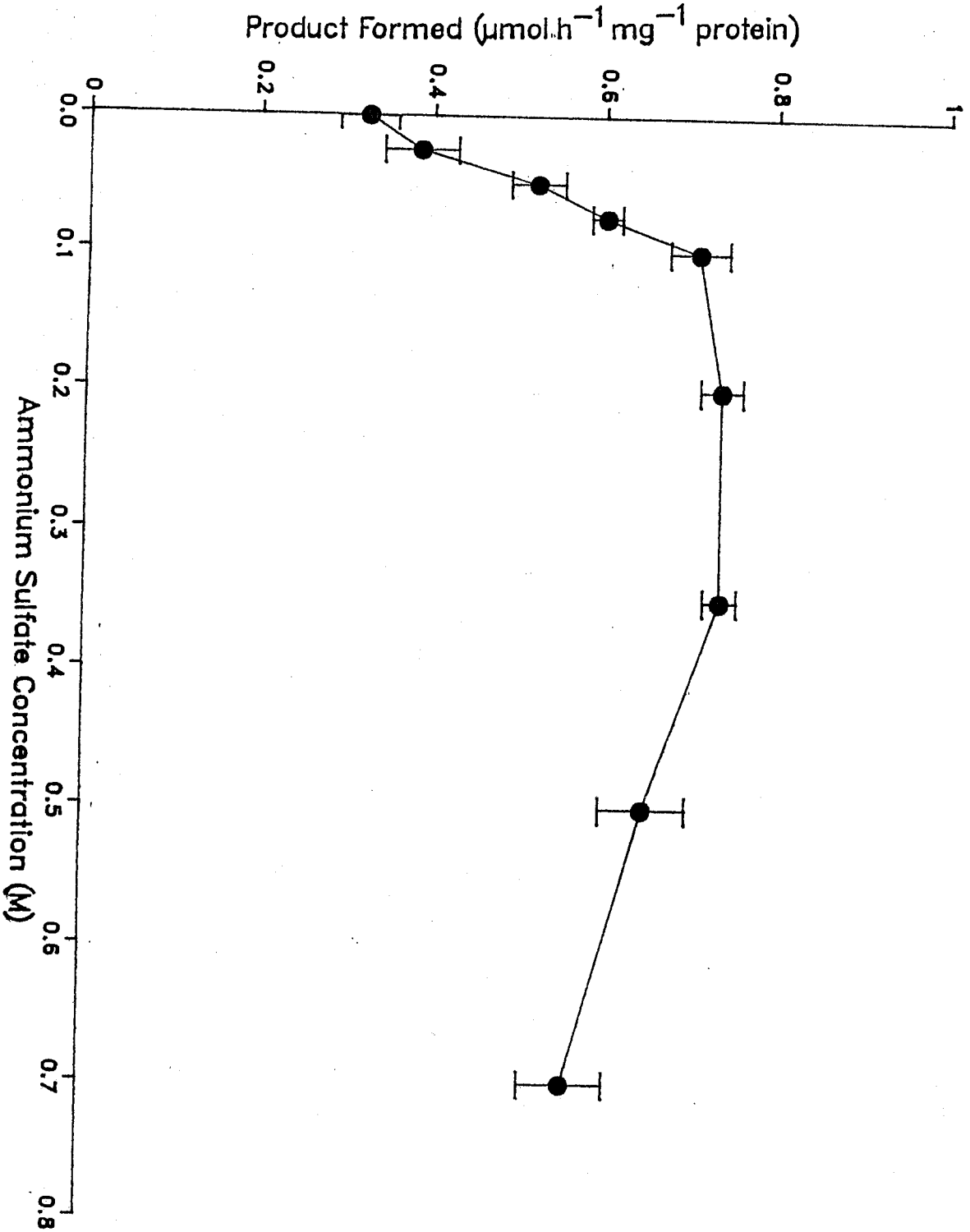

Product Formed (μmol.h^{-1} mg^{-1} protein)
0
0.2
0.4
0.6
0.8
1
Ammonium Sulfate Concentration (M)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8

Table 10. Effect of Different Salts on FPS Activity

Salt	Concentration (M)	FPS Activity μmol h^{-1} mg^{-1} protein.
$(NH_4)_2SO_4$	0.05	0.52
	0.10	0.62
Na_2SO_4	0.05	0.41
	0.10	0.60
KCl	0.10	0.48
	0.20	0.55
NH_4Cl	0.10	0.46
	0.20	0.54

DISCUSSION

In this study, methods for extraction and assay of FPS have been developed. It was found that FPS is a soluble protein and that the crude extract showed a high level of FPS activity (ca. 5 μmol product formed h^{-1} g^{-1} fresh wt) comparable to the *in vivo* rate of floridoside production. Two assay methods, one measuring the formation of floridoside phosphate using gas-liquid chromatography, the other a colorimetric method measuring GLYP dependent formation of UDP gave comparable results. FPS has a high substrate specificity, only UDP-galactose and GLYP can be used as substrates. The FPS catalyzed reaction was linear up to 45 min and proportional to protein concentration in the incubation mixture. The enzyme activity increased with temperature from 0°C to 35°C and was lost above 40°C, and showed a pH optimum of 7.2-8.2. FPS activity was also lost in dilute solutions (protein concentration below 0.5 mg ml^{-1}) or in buffers below 0.2 M ionic strength. Similar to SPS (Huber, personal communication), FPS is stable in concentrated solutions in the presence of 0.3 M ammonium sulfate.

The metabolic pathways in *Porphyra* are still poorly understood. The key substrate, UDP-GAL, could be used for either synthesis of floridoside or synthesis of polysaccharides. It could also be metabolized to UDP-glucose, which can be converted to ADP-glucose and used for the biosynthesis of floridean

starch. Unlike the case in higher plants, the biosynthesis of floridean starch occurs in the cytoplasm in red algae (Meeuse 1962). It is natural to assume that there are controlling points to regulate and coordinate these processes. To carry out these studies, it is imperative that various key enzymes be studied with a view to understanding their roles as biosynthetic or degradative regulators. The establishment of extraction and assay methods for FPS activity opens up the possibility for further studies on other key enzymes.

CHAPTER 5

FIELD STUDIES ON CHANGES IN FLORIDOSIDE LEVEL AND FPS ACTIVITY

MATERIALS AND METHODS

1. Plant materials

This study was conducted from February to May, 1989. *Porphyra perforata* J. Ag. was collected at Stanley Park for two consecutive days at the middle of each month. Six samples were collected at 4 h intervals each day, except early in the morning and late in the evening. More frequent collections were made from 5 AM to 8 AM when both floridoside content and FPS activity showed bigger changes. Less frequent collections were done late in the evening. On the collection dates, the tide level was in medium range. Samples were cleaned of visible epiphytes and epifauna and excessive water was removed by a nylon net. Harvested plants were put immediately into liquid nitrogen to stop metabolism and transferred to -80°C freezer before extraction.

2. Measurement of floridoside

Samples (1 g each) were extracted three times (24 h each) at room temperature with 20 ml absolute ethanol. Alcohol-soluble sugars were separated and quantitated by the GLC procedure described in Section 3 using the trimethylsilylation method.

3. FPS Measurements

Plant samples (4 g each) were homogenized in LN_2 into fine powder. FPS was extracted with 10 ml tricine buffer (50 mM, pH 8.0) with 5 mM DTT, 0.2 M $(NH_4)_2SO_4$, 10 (w/v) PVPP. The extract was centrifuged, desalted, and activity measured as described in Section 4.

RESULTS

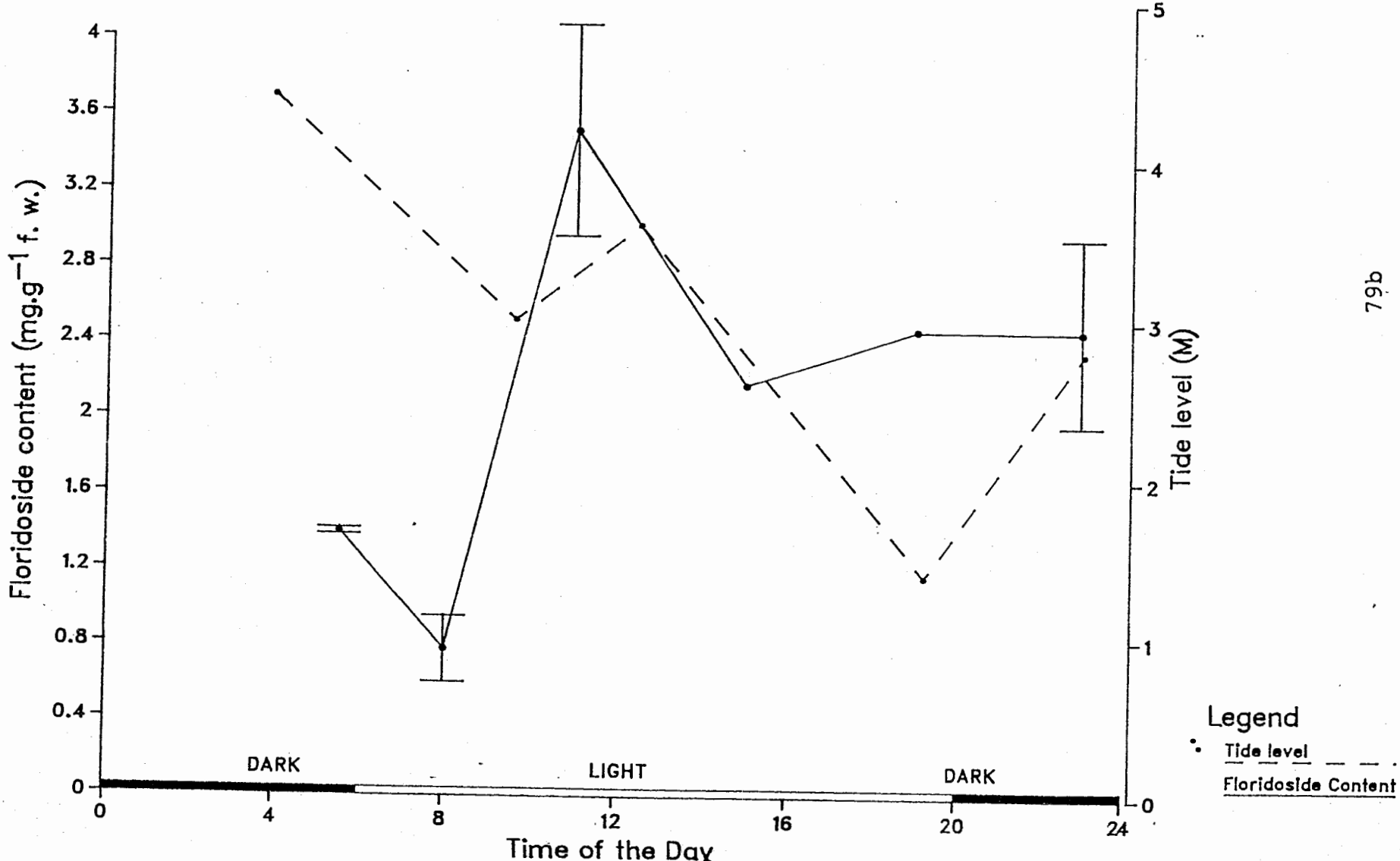
1. Changes in floridoside content

Floridoside levels from field samples increased about 3 fold about three h after sunrise (Fig. 12). The level dropped gradually late in the afternoon. Floridoside was mobilized earlier in the evening and maintained a relatively stable level overnight. This pattern was observed in field collected samples for both March and April (Tables 11 and 12). Samples collected in May showed continuous high levels in the afternoon (Table 13). Floridoside appeared to be affected to a lesser extent by tide level, because some samples collected at high tide showed higher floridoside content.

2. Changes of FPS activity

The activity of FPS in extracts from field samples was highest early in the morning in the samples collected in March and April, maximal FPS activity was attained after sunrise (Fig. 13). Afterwards, FPS activity gradually decreased throughout the day and reached the lowest level early in the evening, the activity at that time was ca. 50% of the peak value. But the difference in FPS activity from field samples was less significant. There was a three h time lag between the peak of FPS activity and that of floridoside content. In May, all samples collected at different times of the day showed similar

Fig. 12 Variation in floridoside content in field samples in over a 24 h period (collected in April).


Floridoside content (mg.g⁻¹ f. w.)
Tide level (M)
Time of the Day
DARK
LIGHT
DARK
0
0.4
0.8
1.2
1.6
2
2.4
2.8
3.2
3.6
4
0
1
2
3
4
5
0
4
8
12
16
20
24
Legend
Tide level
Floridoside Content

79b

Table 11
Floridoside content and FPS activity from field samples collected at different times of the day in March, 1989

Collection Date & Time	FPS Activity $\mu mol\ h^{-1}\ g^{-1}$ f wt	Floridoside Content $mg\ g^{-1}$ f wt
Mar. 16 11:00	8.79±0.12	1.97
15:00	4.49±0.30	1.42
19:30	6.93±0.17	1.50
23:30	7.31±0.13	0.137
Mar. 17 5:30	-	0.604
7:45	9.97±0.18	0.604
11:20	8.50±0.59	2.11
17:00	6.21±0.68	0.711
20:00	5.22±0.24	0.648
Mar. 18 7:00	13.29±0.98	0.562
8:00	7.61±0.45	0.981

Table 12
Floridoside content and FPS activity from field samples collected at different times of the day in April, 1989

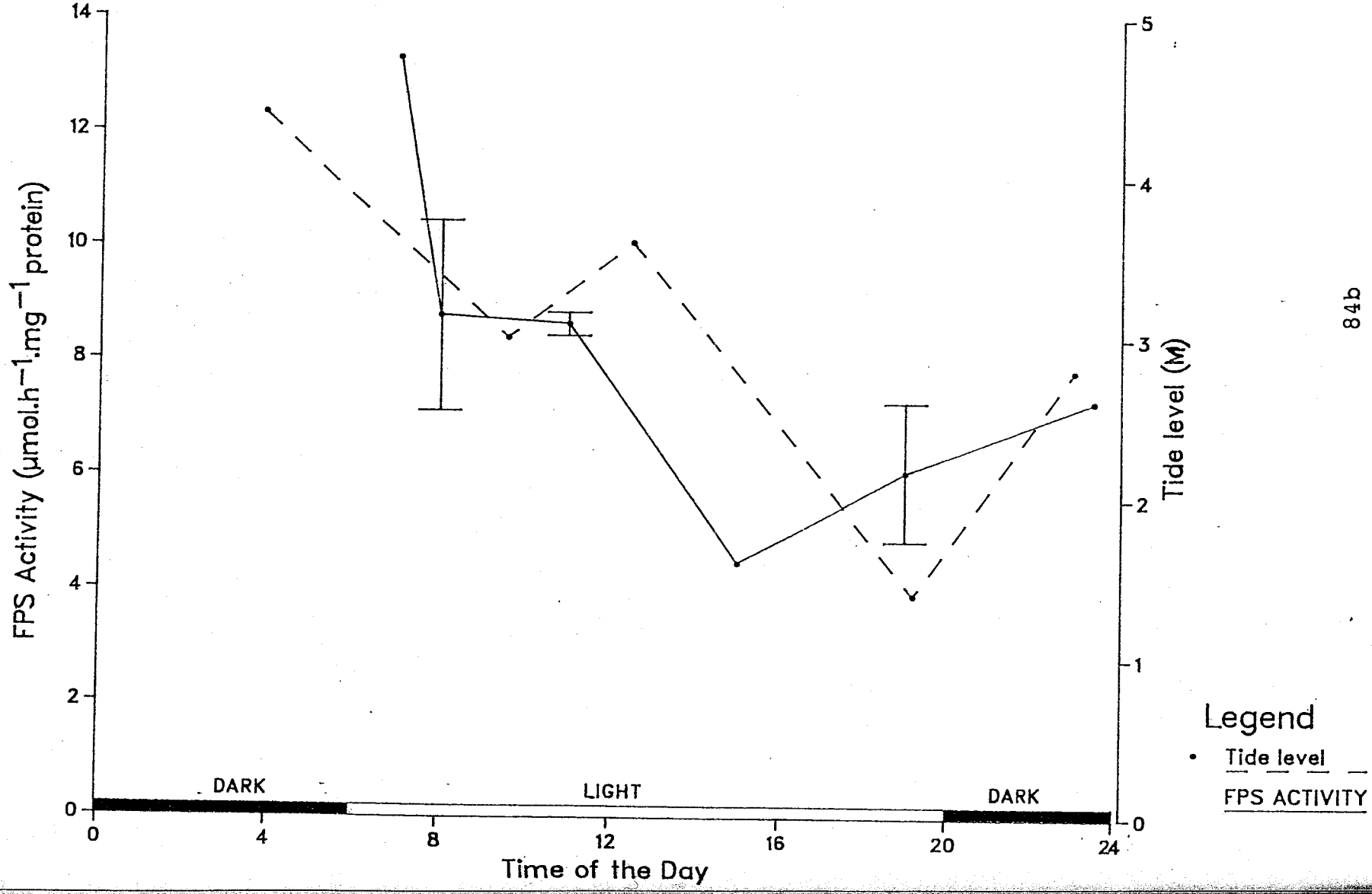
Collection Date & Time	FPS Activity μmol h^{-1} g^{-1} f wt	Floridoside Content mg g^{-1} f wt
Apr. 12 11:00	13.56±0.42	3.13
15:00	11.40±1.49	2.18
19:00	12.36±1.60	-
23:00	12.02±0.41	2.12
Apr. 13 5:30	18.76±1.07	1.39
8:00	11.54±0.27	0.903
11:00	16.42±0.71	3.92
19:00	12.75±0.21	2.48
23:00	10.00±0.19	2.82
Apr. 14 6:00	12.19±0.86	1.41
8:30	11.66±0.92	0.652

Table 13
Floridoside content and FPS activity from field samples collected at different times of the day in May, 1989

Collection Date & Time		FPS Activity μmol h^{-1} g^{-1} f wt	Floridoside Content mg g^{-1} f wt
May 10	11:00	11.40±0.24	3.30
	15:00	10.44±0.72	6.49
	19:00	14.10±1.36	5.72
May 13	5:30	11.17±1.53	1.05
	8:00	11.24±1.03	2.01
	11:00	11.37±0.56	-
	14:30	12.20±0.90	3.02
	19:00	12.12±0.79	4.21
	23:00	15.19±0.30	3.73
May 14	5:00	11.52±0.83	0.576
	8:00	14.55±0.86	1.71

FPS activity (Table 13).

Fig. 13 Variation in FPS activity in field samples over a 24 h period (collected in April)


FPS Activity (µmol.h⁻¹.mg⁻¹ protein)
Tide level (M)
Time of the Day
DARK
LIGHT
DARK
Legend
Tide level
FPS ACTIVITY
84b

DISCUSSION

There were variations in both floridoside content and FPS activity, which indicates that these two parameters do respond to external changes. Changes in floridoside content were much more drastic. The high osmotic pressure created by evaporation, when plants are exposed to air at low tide, seems to have less effect on both floridoside content level and FPS activity. The variations appear more to be diurnal in nature, because the peak values were obtained at around high tide, at that time plants had been submerged in seawater for several h.

In this study, floridoside content peaked at noon, gradually decreased during the afternoon, and stayed at a relatively low level overnight in the samples collected in March and April. These data are somewhat similar to those for sucrose in soybean under short day (7 h light:17 h dark) conditions. In long day (17 h light:7 dark) conditions, a continuous accumulation of sucrose in the afternoon was observed (Huber *et al.* 1984), which is similar to my data on floridoside content in May. One possible explanation for changes in floridoside content is that FPS activity is modulated by the presence or absence of precursors for floridoside synthesis. The transport of substrates for floridoside synthesis, which are photosynthetic products, out of the chloroplast would normally occur in the light; hence, floridoside content is likely to be higher in daylight hours at peak photosynthesis times. In the dark, starch

degradation may produce floridoside, and maintain it at relatively low constant level (Nagashima *et al.* 1969). This proposed control mechanism has also been suggested for SPS (Sicher and Kremer 1984).

The activity of FPS in *Porphyra* increased after sunrise in March and April samples, but the change was less drastic and less variation was observed for samples collected in May. As stated above, floridoside is a photosynthetic product and accumulates in the light when photosynthesis is active. It is not surprising that floridoside content as well as FPS activity correlate strongly with the light and dark period.

Among other factors, nitrogen content would take at least several hours to show an effect (Macler 1986) and is unlikely that it would be the major cause for changes in floridoside content and FPS activity. The seawater temperature did not change very much during the collections and hence it is unlikely that it was a cause for changes in FPS activity. However, in nature the temperature effect on FPS activity could be significant when plants are exposed to air at low tide. Whether temperature will have an effect on floridoside content is not clear. The low floridoside content in the night could be partly due to this effect.

The variations in floridoside content indicate a variation in floridoside pool size in response to physiological stimuli. But this change in pool size is affected not only by floridoside

production rates, but also by degradation of floridoside in the plant *in vivo* according to the metabolic demands. Unfortunately, the degradation pathway for floridoside is not known, and we do not know whether a specific enzyme, like invertase in higher plants, works in red algae. Wiencke and Läuchli (1981) suggested the presence of a floridoside pool in the cytoplasm, that does not exclude the possibility that there may be a minor floridoside pool in the vacuole. If there indeed are two pools of floridoside in red algae, as in the case of sucrose in some higher plants (Sicher *et al.* 1984), the two pools may respond differently to environmental changes.

CHAPTER 6

PARTIAL PURIFICATION AND CHARACTERIZATION OF FLORIDOSIDE PHOSPHATE SYNTHASE FROM *PORPHYRA PERFORATA*

MATERIALS AND METHODS

1. Materials

Porphyra perforata was collected, and treated, and FPS extracted as described in Section 4. All chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo), unless otherwise specified.

2. Methods for purification of FPS

Unless otherwise stated, all steps of enzyme extraction and purification were carried out at 0 to 4°C.

(1). Ion-exchange chromatography

The protein solution from a G-25 column was loaded on a 2.5 X 20 cm DEAE-Cellulose column (DE 52, Whatman) pre-equilibrated with Tricine buffer (50 mM, pH 8.0). The column was first washed with the same buffer until the absorption at 280 nm returned to base line. The column was eluted with a linear gradient from 0 to 0.3 M KCl for one hour at a flow rate of 240 $ml.h^{-1}$. Fractions of 8 ml each were collected. The active fractions were pooled. Ammonium sulfate was added to 80% saturation, equilibrated for 1 h, and centrifuged at 20,000 X g for 30 min. The pellet collected was dissolved in 4 ml Tricine buffer (50 mM, pH 8.0), equilibrated at room temperature overnight, and centrifuged at 12,000 X g for 5 min to clarify the protein

solutions.

(2). HPLC ion exchange chromatography

HPLC was performed with a Waters HPLC system, including two Model 510 pumps, a Model 481 LC spectrophotometer, and a Model 680 Automated Gradient Controller with Waters 740 Data Module. Samples were collected by a Foxy Fraction Collector into 2 ml fractions. A Waters Protein Pak DEAE 5PW ion exchange column was employed. The sample from open column ion exchange was loaded on the column and washed thoroughly. The column was developed at a flow rate of 1 ml min^{-1} with a linear gradient from 0.0 to 0.5 M KCl in 50 mM Tricine buffer (pH 8.0) for 30 min.

(3). Determination of Molecular Weight of the Enzyme

The molecular weight of the partially purified enzyme was estimated by a HPLC gel filtration column (Waters Protein Pak 300 sw) that had previously been calibrated with ferritin (M. W. 440,000), catalase (M. W. 232,000), alcohol dehydrogenase (M. W. 150,000), bovine serum albumin (M. W. 66,000), ovalbumin (M. W. 41,000), and cytochrome C (M. W. 12,400). The column was washed with 0.4 M phosphate buffer (pH 8.0) containing 5 mM GLYP and 5 mM $(NH_4)_2SO_4$. The fraction from open column ion exchange was loaded on the column and developed at 0.5 $ml.min^{-1}$. Fractions of 2 ml each were collected and reconcentrated by 85% ammonium sulphate. The precipitated pellets were resuspended in 0.5 ml Tricine buffer (50 mM, pH 8.0) and assayed.

1. Partial purification of floridoside phosphate synthase

The scheme for purification of FPS from *Porphyra perforata* is summarized in Table 14. A partial purification was obtained using a DE 52 cellulose ion-exchange column and a certain amount of enzyme activity was detected in the fraction eluting at 0.24 M KCl concentration (Fig. 14). The final preparation possessed 3 fold higher specific activity compared to the crude extract with a recovery of about 71%.

The desalted fraction from open column ion exchange was loaded onto an HPLC ion exchange column. A typical elution profile using a continuous KCl linear gradient from 0.1 M to 0.4 M is shown in Fig. 15. Fractions of 1 ml each were collected and assayed for FPS activity. The HPLC ion exchange column has a much higher resolution than the open column, and could separate the fraction from the open column into several components. FPS activity was eluted in a single sharp peak with 50% recovery. Further attempts at purification by an UDP-Agarose affinity column and a Blue A Dyematrex (Amicon, Lexington, MA.) column were unsuccessful. Even if the sample was loaded in the presence of one substrate, GLYP, no FPS activity could be recovered from the column.

Table 14.
Purification of FPS

(Results are shown for one preparation of the enzyme, as described under "Materials and Methods.")

Step	Volume ml	Protein mg	Activity units*	Yield %	Specific Activity units mg^{-1}	Purification (fold)
Sephadex G-25	49	81.9	2964.5	100.0	36.2	1
Ion Exchange	41	23.5	2131.2	71.9	90.7	3
HPLC Ion Exchange	4	0.58	476.1	22.3	820.9	27

* one unit is defined as the amount of enzyme synthesizing one μg floridoside h^{-1}.

Fig. 14 Chromatography of G-25 fraction on a DEAE-cellulose column (see "Materials and Methods"). Protein was measured at UV absorbance of 280 nm. Enzyme activity is presented as the amount of the product formed in μmol h^{-1}.

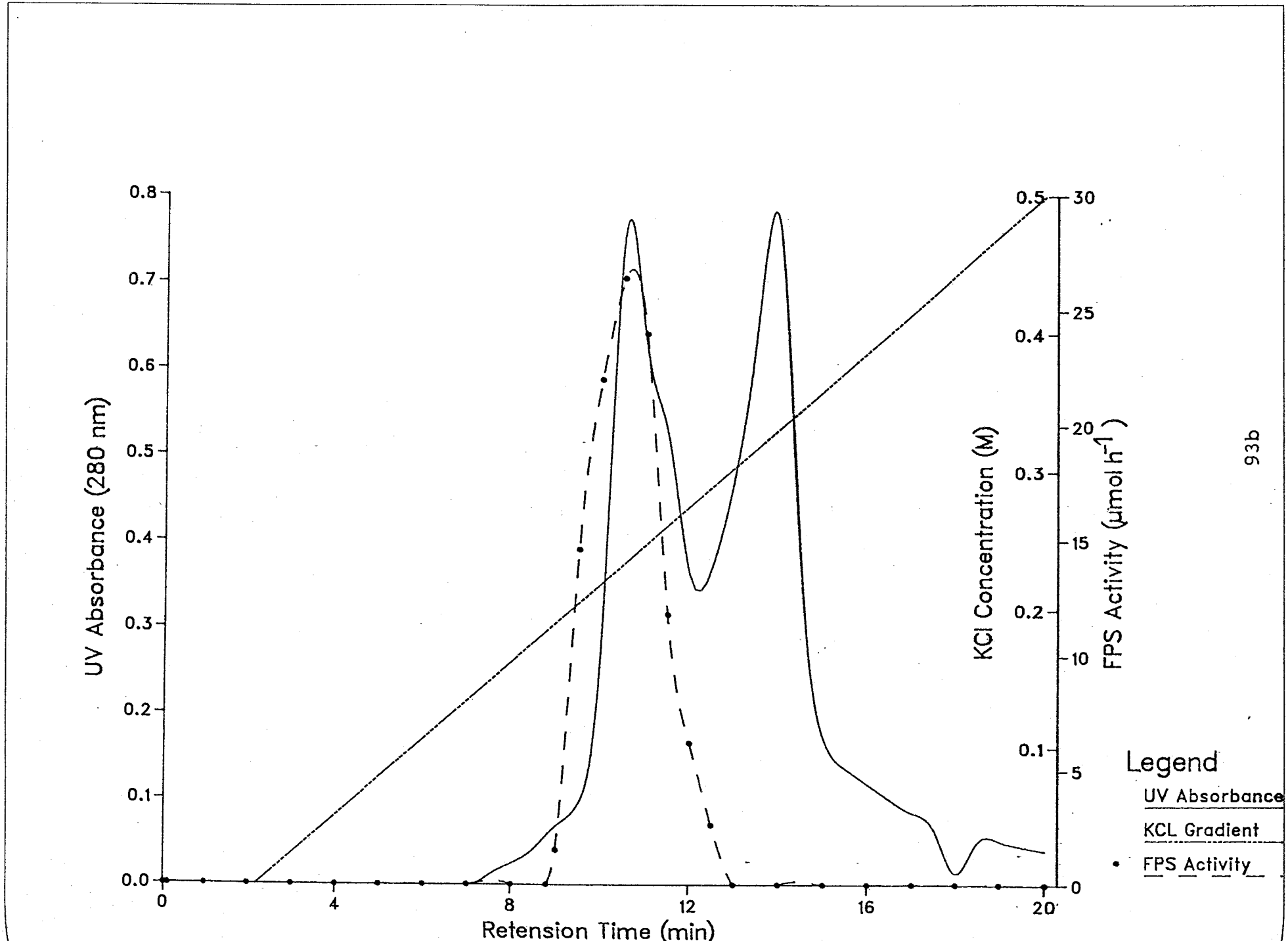
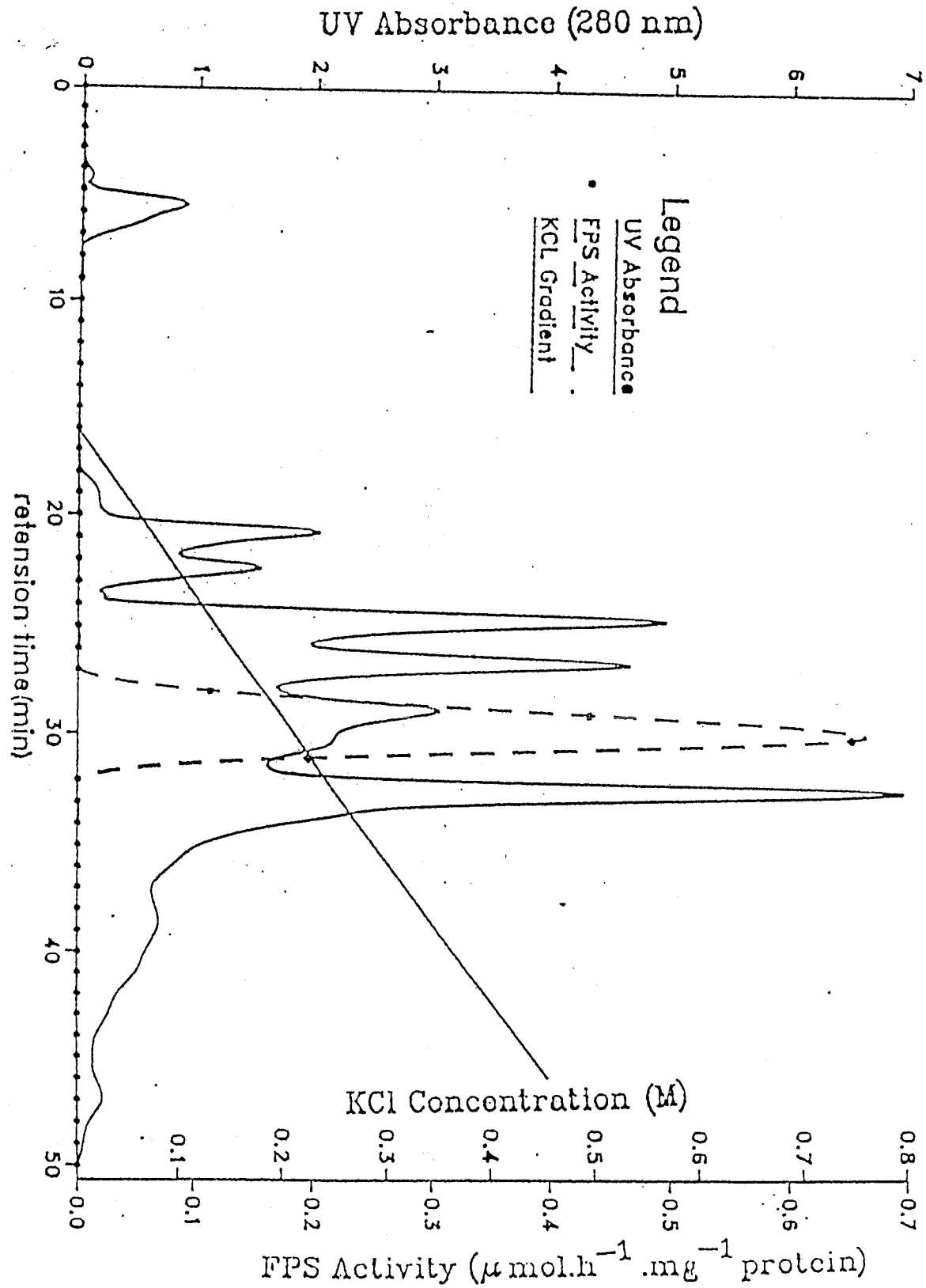

UV Absorbance (280 nm)
KCl Concentration (M)
FPS Activity (µmol h-1)
Retension Time (min)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0
4
8
12
16
20
0
5
10
15
20
25
30
Legend
UV Absorbance
KCL Gradient
FPS Activity
93b

Fig. 15. Elution profile using HPLC ion exchange chromatography.

The active fractions from the open column was pooled, cut with $(NH_4)SO_4$ and the pellet resuspended, clarified, desalted on G-25 column, and loaded onto the HPLC ion-exchange column.


UV Absorbance (280 nm)
Legend
UV Absorbance
FPS Activity
KCL Gradient
retension time (min)
KCl Concentration (M)
FPS Activity (μmol.h⁻¹.mg⁻¹ protein)

2. Determination of Molecular Weight of FPS

The active fraction from the open ion exchange column was loaded onto an HPLC gel filtration column. Fractions of 2 ml each were collected and assayed for FPS activity. The molecular weight of the partially purified FPS, as determined by HPLC gel filtration, was about 140 kD (Fig. 16).

3. FPS enzyme kinetics

The active fraction from the open column ion exchange was used to estimate the kinetic parameters. The FPS activity showed typical hyperbolic curves for both substrates, UDP-GAL and GLYP (Fig. 17, 18). In three separate experiments to determine the kinetic parameters of FPS, the apparent K_m value ranges from 2.3 to 3.4 mM for UDP-GAL and 8 to 12 mM for GLYP, as determined by Eadie-Hofstee plots for both substrates and confirmed by an EZ-FIT microcomputer program (Perrella 1988). Representative examples for both substrates are shown in Fig. 19 and 20, respectively. The apparent V_{max} for these enzyme preparations was ca. 2 μmol h^{-1} mg^{-1} protein.

4. Some properties of FPS

Fig. 16. Calibration of FPS molecular weight. The molecular weight of the protein was calibrated against protein standards.

The protein standards used were

Ferritin	440 Kd
Catalase	232 Kd
Alcohol dehydrogenase	150 Kd
BSA	66 Kd
Ovalbumin	41 Kd
Cytochrome C	12.4 Kd

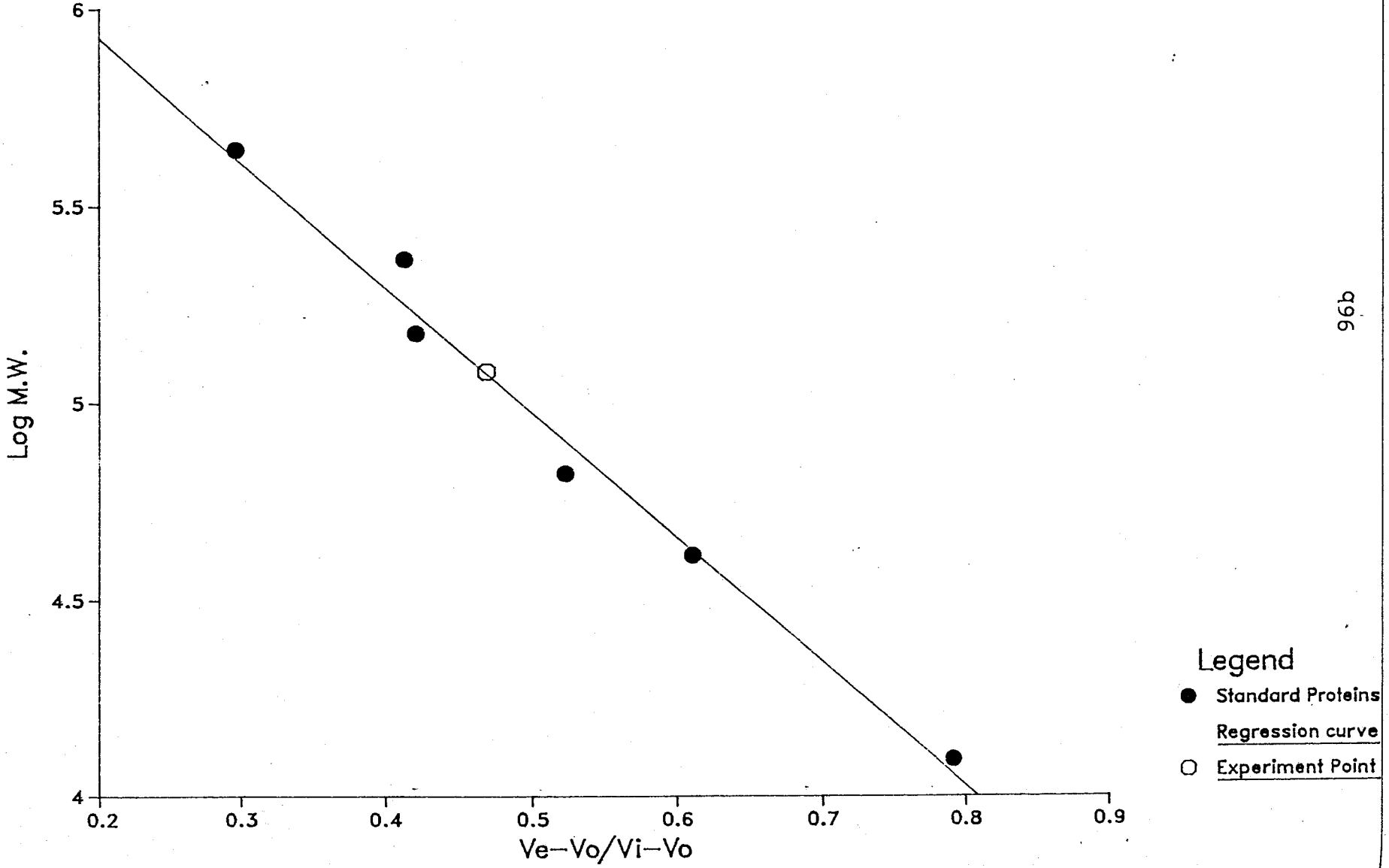

6
5.5
5
4.5
4
Log M.W.
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
Ve—Vo/Vi—Vo
Legend
Standard Proteins
Regression curve
Experiment Point

Fig. 17. FPS activity at Different Concentrations of UDP-GAL (GLYP concentration is 40 mM)

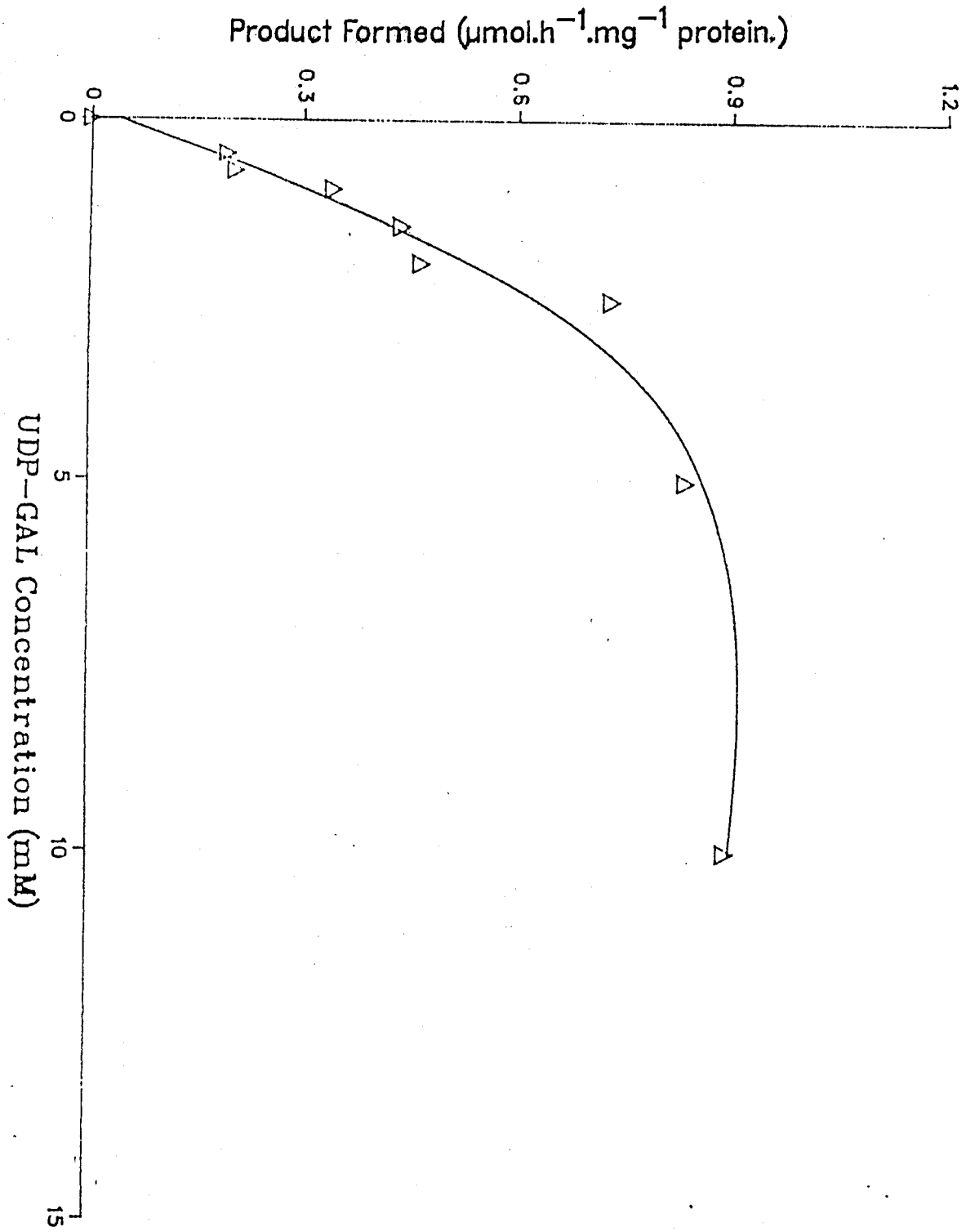

Product Formed (µmol.h^{-1}.mg^{-1} protein.)
0
0.3
0.6
0.9
1.2
UDP-GAL Concentration (mM)
0
5
10
15

Fig. 18. FPS activity at Different Concentrations of GLYP (UDP-GAL concentration is 10 mM)

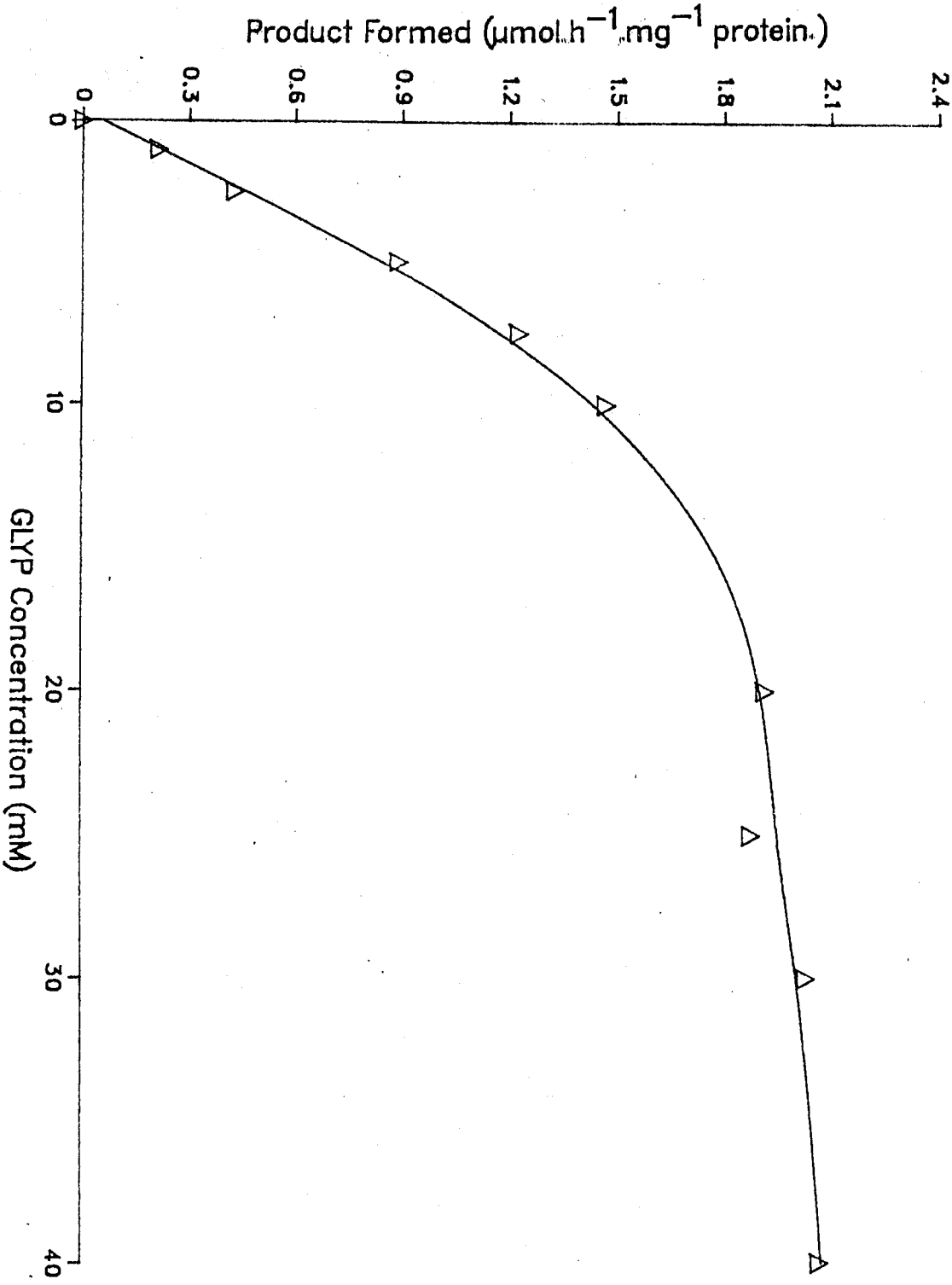

Product Formed (μmol.h^{-1}.mg^{-1} protein.)
0
0.3
0.6
0.9
1.2
1.5
1.8
2.1
2.4
GLYP Concentration (mM)
0
10
20
30
40

Fig. 19. An Eadie-Hofstee Plot of UDP-GAL Kinetics

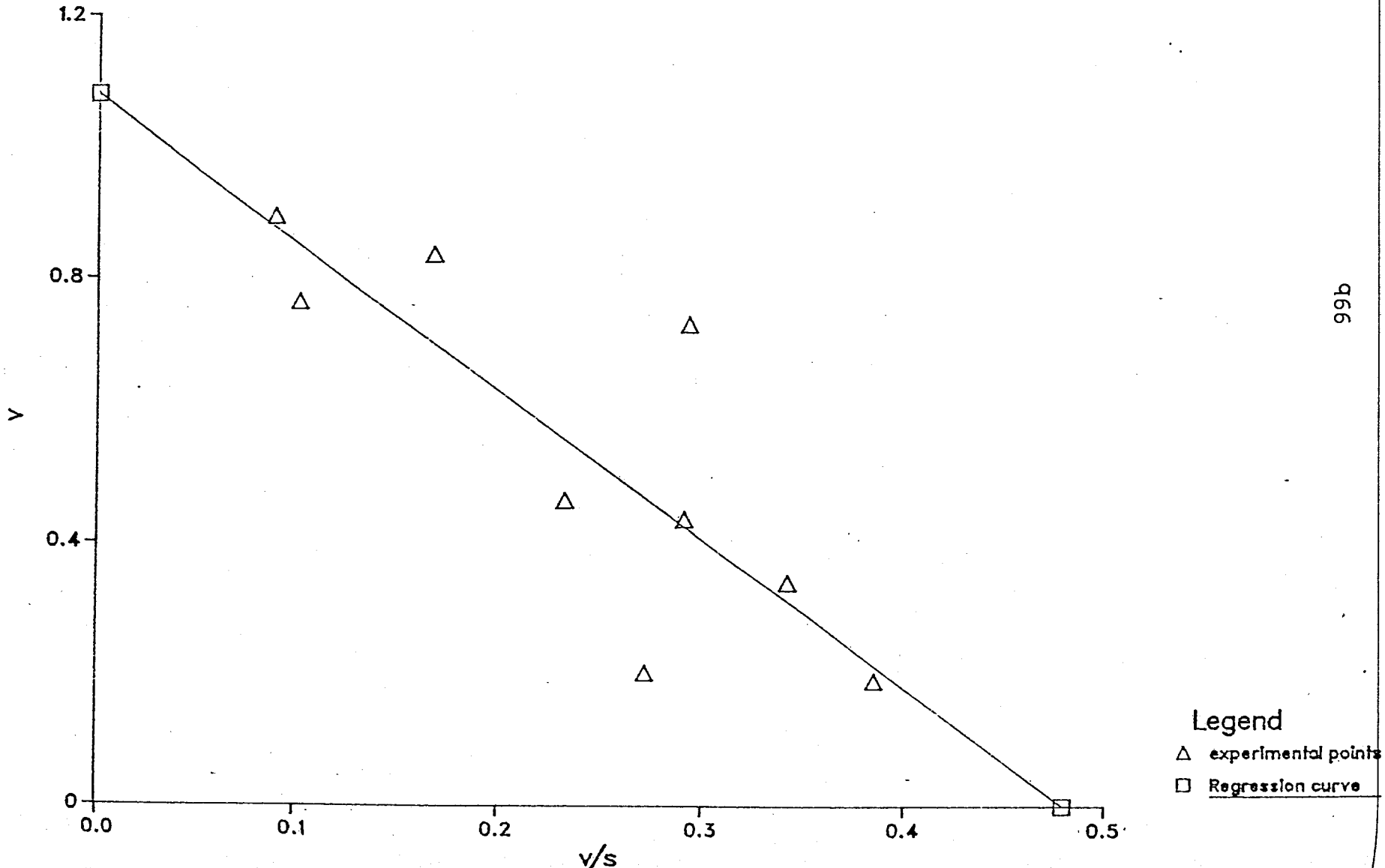
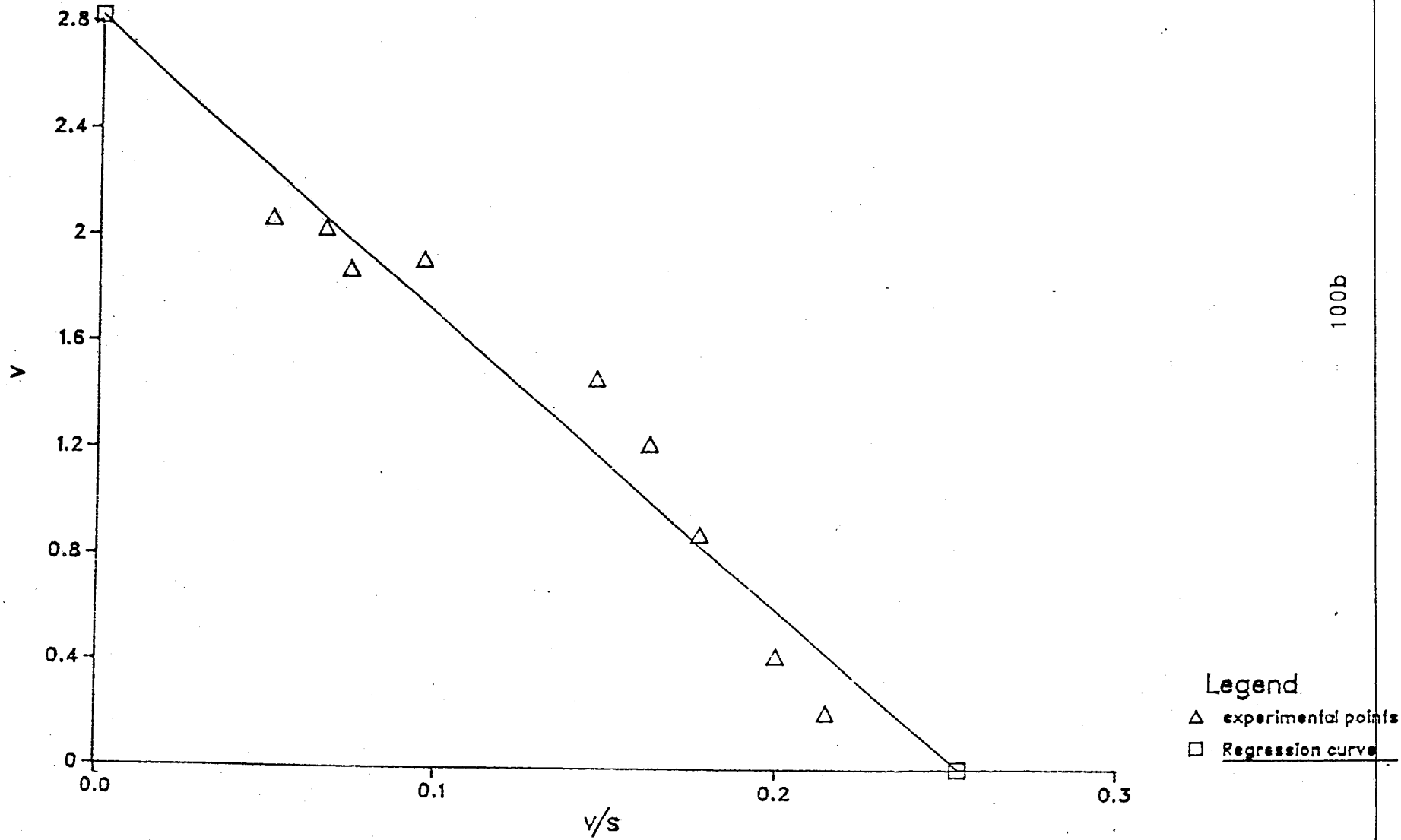

1.2
0.8
0.4
0
V
0.0
0.1
0.2
0.3
0.4
0.5
V/s
Legend
experimental points
Regression curve

Fig. 20. An Eadie-Hofstee Plot of GLYP Kinetics

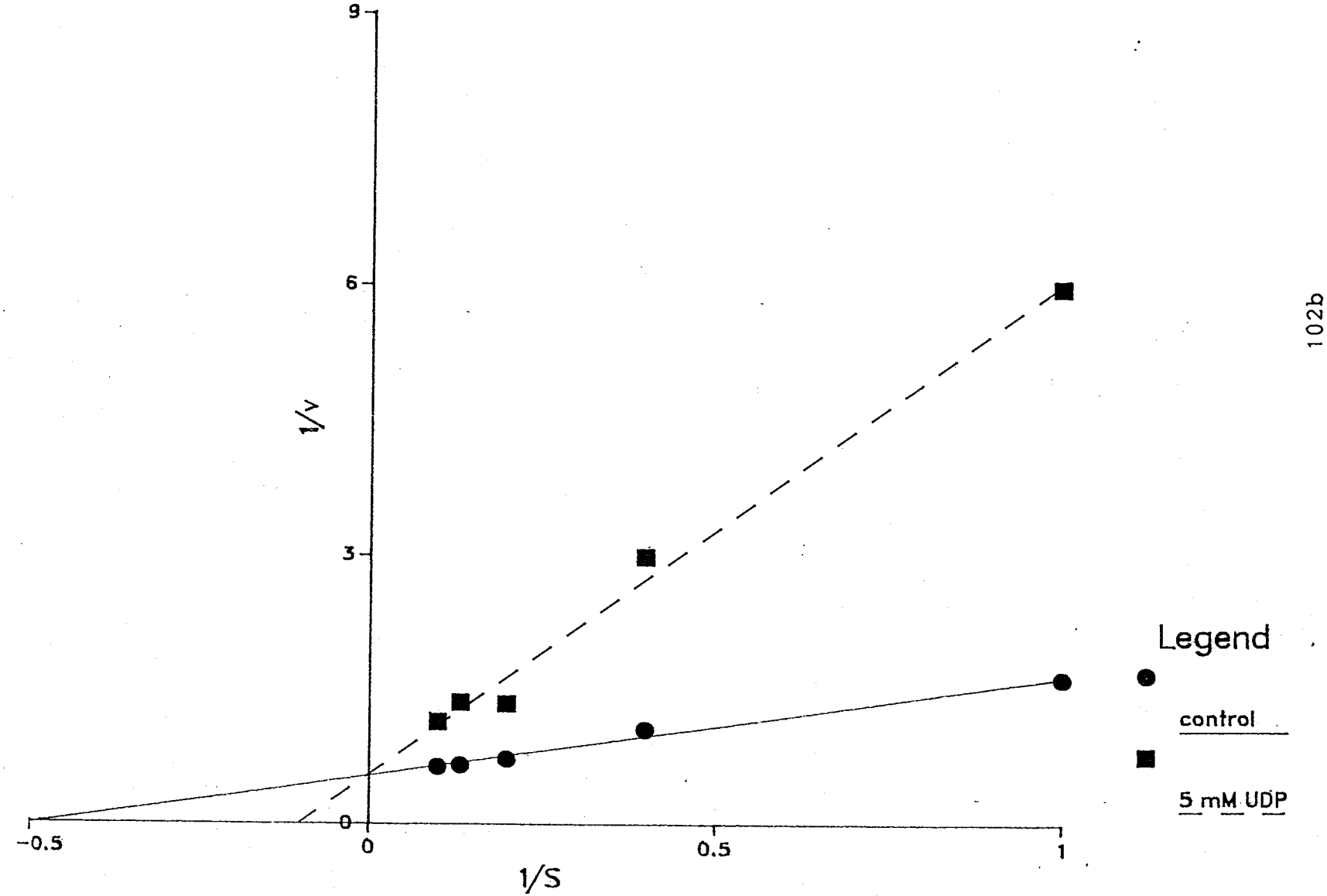

2.8
2.4
2
1.6
1.2
0.8
0.4
0
V
0.0
0.1
0.2
0.3
V/s
Legend
experimental points
Regression curve

100b

(1). Effect of metabolites

Metabolites including glycine, alanine, cysteine, aspartate, asparagine, glutamate, glutamine, citrate, succinate, malate, oxaloacetate, phosphoenolpyruvate, pyruvate, glucose-1-phosphate, glucose-6-phosphate, glucose-1, 6-diphosphate, fructose-1-phosphate, fructose-6-phosphate, fructose-1, 6-diphosphate, fructose-2, 6-diphosphate, galactose, galactose-1-phosphate, galactose-1, 6-diphosphate, galactose-6-phosphate, AMP, ADP, and ATP, each at a concentration of 5 mM, had no significant effect on the activity of the enzyme. However, enzyme activity was inhibited by UDP (5 mM). UDP acted competitively against UDP-GAL. The inhibitory effect of UDP on GLYP was not studied. (Fig. 21).

Fig 21. Effect of UDP on FPS catalyzed reaction.



102b

DISCUSSION

A protocol including sequential open column DE 52 ion-exchange, and HPLC ion-exchange chromatography was used to prepare a partially purified FPS from *P. perforata*. This protocol produced a specific activity of 821 units mg^{-1} protein with a 20% yield and a 30 fold purification. The enzyme preparation was not homogeneous, as assessed by silver-stained SDS-PAGE.

Although a number of methods were examined for further purification of FPS, they were hampered by the relatively low stability and recovery of purified fractions. HPLC ion exchange chromatography could purify FPS about 10 fold, but the purified fraction was not stable. The activity could not be maintained overnight even in the presence of one substrate, GLYP, and 0.5 M ammonium sulfate. Similar problems were encountered with UDP-Agarose and Blue A affinity columns. The exact nature of this protein denaturation is not clear, but from previous protein dilution experiments, it appears that the more purified protein fractions are diluted below the required protein concentration to maintain stability (see Section 4, protein dilution test). The most likely explanation for this phenomena is that FPS is an oligomer and dissociates into inactive subunits. This assumption is supported by two other observations: 1. it was found that no cofactor (maybe with the exception of Mg^{2+}, see below) was required for the FPS catalyzed

reaction; and 2. addition of protease inhibitors, e.g. PMSF, did not show a detectable improvement in enzyme activity. But the evidence from hyperbolic kinetics observed for the FPS catalyzed reaction is against this assumption. More work is needed to clarify the situation. Many UDP-glycosyl transferases from animal systems have been shown to require Mn^{2+} or Mg^{2+} as cofactors or activators for the reactions (Abe *et al.* 1988, Elices and Goldstein 1988, and Navaratnam *et al.* 1988). However, no cofactor was required for sucrose phosphate synthase (Huber, personal communication), and my results with FPS are not clear. In my studies, UDP was found to inhibit the FPS activity, an inhibition that was partly overcome by addition of Mg^{2+}, this result could be due to the effect of Mg^{2+} as a cofactor or due to the possibility that Mg^{2+} forms complexes with UDP and thus partly eliminates the inhibitory effect of UDP.

The regulation of FPS activity *in vivo* is an important question, because floridoside is one of the major photosynthetic and reserve products. The regulation of floridoside biosynthesis is likely to involve interactions between other reserve carbohydrates (e.g. floridean starch) on the one hand, and carbon flow during photosynthesis on the other. There are at least three possible levels for the mechanism of regulation for the biosynthesis of floridoside: 1. biosynthesis and degradation of FPS; 2. allosteric regulation or covalent modification; 3. changes in concentrations of substrates and products. It is known that SPS can be regulated in several ways by all three

levels. In sucrose biosynthesis, it was shown that one mechanism involves allosteric control, with glucose-6-phosphate acting as an activator and P_i as an inhibitor (Doehlert and Huber 1983, 1984). Allosteric control by sugar phosphate does not seem to apply to FPS catalyzed reaction because sugar phosphates did not affect the activity of the enzyme preparations (see Results, this Section). The presence of isozymes in different proportions could affect enzyme activity. However, ion exchange chromatography, which is one of the common methods to distinguish different isozymes, did not reveal the presence of isozymes in the FPS preparation. This result suggests that either there are no FPS isozymes in *P. perforata*, or the difference in charge among different isozymes is not big enough to separate them by ion exchange chromatography.

Covalent modifications of an enzyme can affect enzyme kinetic parameters and enzyme activity. However, no evidence has been found in the experiments conducted so far that indicates that there is a change in the kinetic parameters of the FPS catalyzed reaction.

The *de novo* synthesis of the enzyme in response to regulation is a possibility for floridoside synthesis regulation. This could explain the fluctuation of FPS activity in field collected samples (see section 5), as no significant change was found for the kinetic parameters of FPS.

As in all enzymes, changes in concentrations of substrates and products will affect FPS activity. Accumulation of a product, such as UDP, strongly inhibited FPS activity and increased UDP-GAL concentrations can partly eliminate this inhibitory effect (see results). However, accumulation of floridoside, the end product in the pathway, did not show any effect on FPS activity.

In higher plants, the principal end products of photosynthesis are sucrose and starch. Triose phosphates produced in the chloroplasts during photosynthesis are the starting point for sucrose synthesis, and represent an important branch point in photosynthetic metabolism. Some of the triose phosphates are retained in the Calvin cycle to regenerate RuBP which is the acceptor for further CO_2 fixation. The surplus triose phosphates are partitioned between sucrose and starch. The transport of triose phosphate for sucrose synthesis out of the chloroplast normally occurs in the light and during the night when starch degradation is reported to take place (Sicher and Kremer 1984). Since starch is presumably synthesized when the conversion of triose phosphates to sucrose in the cytosol is being restricted, some of the surplus triose phosphates may be retained in the chloroplast for conversion to starch (Stitt *et al.* 1984). The first irreversible reaction during the conversion of triose phosphates to sucrose in the cytosol is catalyzed by the cytosolic fructose 1,6-bisphosphatase (Foyer *et al.* 1982). This enzyme is inhibited by fructose 2,6-bisphosphate (Cseke *et*

al. 1982), a signal metabolite. Stimulation of the cytosolic fructose 1,6-bisphosphatase would lead to increased production of hexose phosphates. It has been shown that SPS is stimulated by glucose 6-phosphate and inhibited by inorganic phosphate (Doehlert and Huber 1983). It appears that, by altering the activity of the fructose 1,6-bisphosphatase, an alteration in the concentration of glucose 6-phosphate results which in turn modulates the activity of SPS. In this way, the rate of withdrawal of triose phosphates from the chloroplasts is coupled to the rate of sucrose synthesis.

In red algae, the dark reactions of photosynthesis are similar to those in higher plants (Bean and Hassid 1955). However, there appear to be two major differences in photosynthate partitioning between higher plants and red algae:

1. Since floridean starch is synthesized in the cytosol, triose phosphates transported from chloroplasts will provide carbon skeletons for both synthesis of floridoside and floridean starch in the cytosol.
2. UDP-GAL is the substrate for floridoside synthesis (Bean and Hassid 1955), whereas ADP-glucose is the most efficient glucosyl donor in starch synthesis (Nagashima *et al*. 1971). Since both UDP-GAL and ADP-glucose are produced from hexose phosphates, the regulation in partitioning of photosynthate into two major products, floridoside and floridean starch would occur after formation of hexose phosphates. Nagashima *et al*. (1969) reported that in *Serraticardia maxima* ^{14}C radioactivity was most rapidly

incorporated into floridoside in the light in 1 h. After 3 h illumination, however, incorporation of the radioactivity in floridean starch has been gradually increased. This implies that when the conversion of hexose phosphate to floridoside is being restricted in the cytosol, some of the surplus hexose phosphates are used for conversion to floridean starch.

It has been suggested that SPS is the rate-limiting enzyme for sucrose formation in higher plants and that a high activity of SPS is associated with greater synthesis of sucrose and reduced accumulation of starch (Huber and Israel 1982). The synthesis of floridoside phosphate could be regulated by FPS in a similar manner, and thus control the photosynthetic formation of floridoside and floridean starch in *Porphyra*.

CHAPTER 7

CONCLUSIONS

Porphyra is one of the most cultured and consumed seaweeds in Japan and China. It has a high protein and carbohydrate content. However, little is known about the metabolic pathways and control mechanisms in *Porphyra* (red algae in general). The chemical structures of floridoside and isofloridoside were established in the 1930's, and the biosynthetic pathway for floridoside was established in the early 50's. The production of the major photosynthetic product, floridoside, was shown to vary in response to external factors. But, not much work had been done on the biochemical aspect of floridosides and the enzymes responsible for the synthesis of floridoside had not been studied.

In this thesis, results for chemical characterization of floridosides, extraction and assay of FPS, and partial purification and characterization of FPS are presented. Variations of both floridoside content and FPS activity in field samples were also investigated.

Studies on floridosides in *Porphyra* require separation and identification methods which are reliable and easy to use. The separation of floridosides involves isomers of floridoside with only a linkage difference in the molecules and is quite difficult. In the present study, a chemical analysis was done on various isomers of floridoside from *Porphyra perforata*. Separation methods for these isomers of floridoside were established. It was found that ^{13}C-NMR provided the most powerful method for identification of each isomer. All these

isomers showed their characteristic peaks on ^{13}C-NMR spectra. These low-molecular-weight carbohydrates can be separated, identified, and quantified by GLC-MS using a combination of different derivatizing methods. The GLC method provides an easy tool for routine analysis. Even though not much is known about the physiological function(s) of isofloridoside in *Porphyra*, it was found in large quantities in the field samples collected. Furthermore, the content of each isomer of isofloridoside was shown to vary considerably in different samples collected at different times of the year.

The enzyme responsible for the biosynthesis of floridoside, FPS, was extracted and assayed. The extraction methodology was systematically studied and compared while changing a different extraction component each time. Two assay methods were established to measure FPS activity. The results from these two methods were comparable. Furthermore, the UDP assay method can be used to estimate GLYP-independent metabolism of UDP-GAL. Extracted FPS activity from *Porphyra perforata* fell in the range of 5-7 μmol h^{-1} g^{-1} f wt, which is in the same range as the *in vivo* rate of floridoside biosynthesis. FPS was partially purified and characterized. A specific fraction from DEAE ion exchange chromatography showed enhanced specific activity. The reaction catalyzed by FPS was linear with time and protein concentration under chosen assay conditions. The enzyme was found to be a soluble protein with a molecular weight of ca. 140 Kd, as estimated by HPLC gel filtration chromatography. FPS has

a high substrate specificity, a broad pH range for activity, and requires high protein concentration and 0.2 M ammonium sulfate to stabilize it. The apparent K_m values were 3 and 10 mM, for UDP-GAL and GLYP, respectively, which are comparable to K_m values for SPS catalyzed reaction. One product, UDP, was shown to inhibit FPS catalyzed reaction competitively; this inhibitory effect can be partially eliminated by increase concentration of UDP-GAL.

An analysis of field-collected samples showed that both floridoside content and FPS activity varied at different collection times in the 24 h period and with the season , which indicates that they are responding to changes in external conditions.

The control mechanism for floridoside biosynthesis is not yet clear. Data obtained so far indicate that concentrations of substrates for FPS is one way of controlling its biosynthesis. If FPS is an oligomer, as proposed in Section 6, the dissociation and association of subunits may also affect the FPS activity *in vivo*.

In this thesis, the biosynthetic and physiological aspects of floridosides in *Porphyra* were examined. The methodologies for identification and characterization of different isomers of floridoside in *Porphyra* may be useful in future analyses of these compounds in red algae. Since *Porphyra* is an important commercial crop, these methods may also be useful for assessment

of the harvest quality and improvement of cultivation methods so as to produce *Porphyra* with high floridoside content. Extraction, assay, and characterization of FPS from *Porphyra* is one of the most important requirements for understanding the biosynthesis of floridoside and its control mechanisms in red algae. The *in vitro* enzymological work as reported in this thesis is likely to provide clues to the *in vivo* partitioning of photosynthetic products between two major storage carbohydrates, floridoside and floridean starch. By studying FPS and other key metabolic enzymes, we can also understand the interactions and the relationships among biosynthesis and degradation of other major cellular components, e.g. protein, amino acids, cell wall polysaccharides. Since floridoside is the major photosynthetic product for most red algae, many of the results presented here for *Porphyra* could be applicable to other red algae. It is expected that the data presented in this thesis will help us understand some basic biological processes in red algae, which in turn may provide useful information for future commercial cultivation of *Porphyra* and many other red algae.

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