Pergamon

PII: S0031-9422(97)00284-7

Phytochemistry, Vol. 46, No. 2, pp. 209–213, 1997

Printed in Great Britain
0031-9422/97 $17.00+0.00

IDENTIFICATION OF THE FORMS OF BORON IN SEAWEED BY ^{11}B NMR

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(*Received in revised form* 24 *February* 1997)

Key Word Index—*Chlorophyta*; *Rhodophyta*; *Phaeophyta*; algae; boric acid; boron complex; ^{11}B NMR; mannitol.

Abstract—Free and complex forms of accumulated borate in seaweeds were identified by ^{11}B NMR. The NMR spectra of green and red algae showed boric acid peaks mainly. In contrast, brown algae gave two distinctive peaks predominantly containing borate diesters. To identify the moiety coupled to borate, borate complexes with compounds containing vicinal hydroxy groups, such as mannitol, laminarin and alginic acid, were prepared and examined. The results strongly indicated that mannitol formed the main diester complexes. The signal of the complexes was decreased rapidly after storage. Fresh brown algae, therefore, contain large amounts of B-carbohydrate complexes as do higher plants. © 1997 Elsevier Science Ltd

INTRODUCTION

After the discovery in 1910 [1] that boron is an essential element for higher plants, its physiological role has been the subject of a number of investigations. Recently, it has been suggested that in animals boron plays a regulatory role in the metabolism of minerals. An example of this is its role in the metabolism of calcium in bone [2–4]. In the case of seaweed and other lower plants there have been many reports on the elemental boron content, however, the role and the form of boron *in vivo* has not yet been elucidated. Seaweeds are known for their richness in minerals including boron [5], and many species are consumed as food, especially in eastern Asia. Clarifying the form of boron *in vivo* is seen as a first step in determining the role of boron in lower plants. In this study we have investigated the ^{11}B NMR spectra of three major kinds of algae, i.e. red, green and brown algae.

RESULTS AND DISCUSSION

In vivo ^{11}B NMR spectra

The spectra of red, green and brown algae are shown in Fig. 1. The chemical shifts were referenced to external boric acid as 0.0 ppm at pH 6.0. As shown in Fig. 2, the shift for boric acid was between 0 and −2 ppm in the range of physiological pHs.

Each spectrum of all of the examined seaweed showed a peak at *ca* 0 ppm which was assigned to boric acid (Fig. 1). In the spectra the red alga *Grateloupia turuturu* (Fig. 1, R2) and the green alga *Ulva fasciata* (Fig. 1, G1) two small peaks were observed in the range of −9 to −15 ppm in addition to boric acid peaks. The other green alga, *Ulva pertusa*, afforded only the broad peak at *ca* −11 ppm other than the one at 0 ppm (Fig. 1, G2).

The spectra of brown algae were quite different from those of red and green algae. As shown in Fig. 1, the boron element existed in at least three forms in the stipe and two forms in the blade of the brown algae, examined. An intense peak appeared at *ca* −10 ppm in each spectrum measured for both *Undaria pinnatifida* and *Laminaria japonica* (Fig. 1, B1b, B1s, B2b, B2s) independent of the moiety. Of the four spectra recorded, those of the stipes had small shoulder peaks accompanying the predominant peaks (Fig. 1, B1s, B2s). These signals at *ca* −10 and −14 ppm correspond to the borate diester forms and monoester forms, respectively, according to the assignment spectra in higher plants [6]. The brown algae contain diesters specifically, while boric acid is the dominant species in the red and the green algae. Based on the chemical shifts of boric acid which were observed in the vicinity of 0 ppm, it is suggested that the cellular boric acid is at a pH less than 6.5.

The ligand of borate in brown algae

The candidates for complex formation with borate in brown algae should have free vicinal hydroxyl

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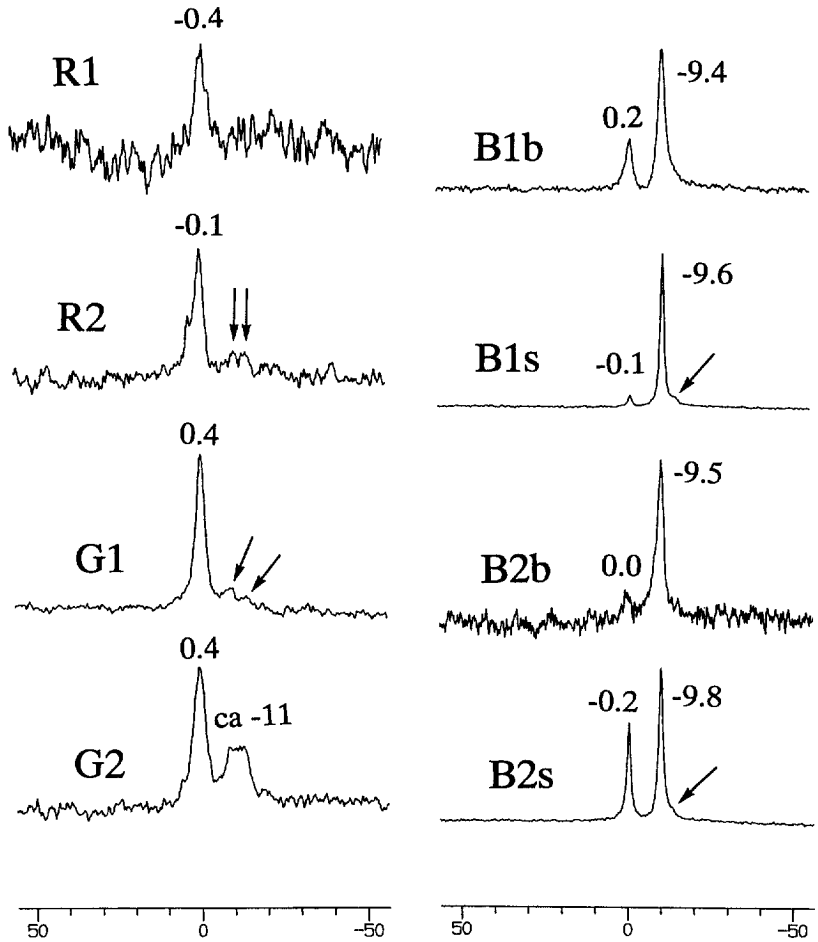

Fig. 1. *In vivo* ^{11}B NMR spectra of red, green and brown algae: R1, *Gloiopeltis tenax*; R2, *Grateloupia turuturu*; G1, *Ulva fasciata*; G2, *Ulva pertusa*; B1b, blade of *Undaria pinnatifida*; B1s, stipe of *Undaria pinnatifida*; B2b, blade of *Laminaria japonica*; B2s, stipe of *Laminaria japonica*. The spectra were plotted using the same *Y*-axis gain. Small peaks are indicated by arrows.

groups, such as mannitol, laminarin and alginic acid. Mannitol and laminarin are the main products of photosynthesis, and alginic acid is a cell wall constituent of brown algae. In fact, the levels of mannitol, laminarin and alginic acid are very high, that is, 6–26 [7], 7–35 [8] and 13–50% [8], respectively. The spectra of boron when borate is added to mannitol at various pH values are shown in Fig. 3. In contrast to the result that the chemical shift of boric acid altered with pH, that of the diester was almost constant. The monoester and diester peaks of the B-mannitol complex were observed at *ca* −14 and −10 ppm, respectively. The spectral pattern of boron with laminarin and alginic acid at pH 8.6 and 7.1, respectively, are shown in Fig. 4. No peak at *ca* −10 ppm was observed either in the spectrum of B-laminarin or that of B-alginic acid. The borate complex of laminarin and alginic acid gave small peaks at −13.7 and −6.3 ppm, respectively, attributed to monoester and diester, respectively. Further, a small peak of borate was detected at −18.2 ppm, which was probably due to inhomogeneities caused by aggregation of the polysaccharides. Therefore, the borate ligand in brown algae is most probably mannitol. The signal superiority of borate diester to monoester in the spectra of brown algae suggests that the content of total boron is extremely low compared with those of dihydroxy compounds, so that the equilibria shift to diester formation.

B content of seaweeds

To investigate the relationship between boron content and the ratio of the B complexes, the quantity of boron in seaweed was measured by inductively coupled plasma atomic emission spectrometry (ICP-AES) after microwave digestion (Table 1). The boron content of *G. tenax* was the lowest (54 $\mu g\ g^{-1}$ of the dry wt), whereas the stipe of *L. japonica* contained the highest amount of boron (331 $\mu g\ g^{-1}$ of the dry wt)

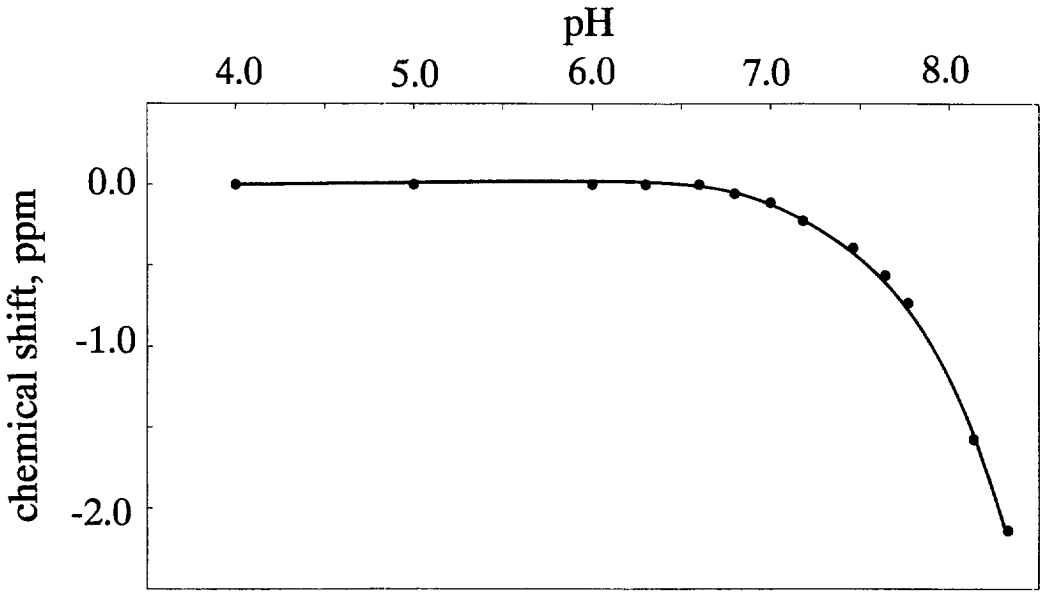

Fig. 2. The pH-titration curve of H_3BO_3. The chemical shifts of the H_3BO_3 under various pH values were plotted. The pH was adjusted with 1, 5 M NaOH or 1 M HCl.

and the blade contained half as much. These values were generally higher than levels in most higher plants.

Although, the profiles of the ^{11}B NMR spectra differed from each other between brown and red algae, the boron contents of *G. tenax* and *U. pinnatifida* were approximately equivalent. Thus diester formation depends not on the quantity of boron but also on the amounts and species of the ligand.

Borate complexes after harvesting

The blade of *L. japonica* was kept frozen (−20°) for two or four days or at room temperature (22°) after harvesting. The ^{11}B NMR spectra of these samples are shown in Fig. 5. With the passage of time, the intensity of the diester peak (*ca* −10 ppm) decreased whereas that of boric acid peak (*ca* 0 ppm) increased. The diester disappeared after 24 hr even when the blade was stored in a refrigerator at −20°. These results suggest that the B-complex of the carbohydrate that exist in fresh brown algae can be degraded rapidly.

Many hypotheses about the physiological role of boron have been discussed, for example, involvement in sugar transport, cell wall synthesis, lignification, and so on [9]. Our study of boron forms in seaweed offers the key to an aid of clarifying the physiological function of boron in the plant kingdom.

Table 1. Boron contents ($\mu g\ g^{-1}$, dry wt) in seaweed

Seaweed	Tissue	Content
Chlorophyta (green algae)		
Ulva fasciata		81
Ulva pertusa		116
Rhodophyta (red algae)		
Grateloupia turuturu		68
Gloiopeltis tenax		54
Phaeophyta (brown algae)		
Undaria pinnatifida	blade	87
	stipe	101
Laminaria japonica	blade	168
	stipe	331

EXPERIMENTAL

Algae and reagents. The two green algae (*Ulva fasciata, U. pertusa*), two brown algae (*Laminaria japonica, Undaria pinnatifida*) and two red algae (*Grateloupia turuturu, Gloiopeltis tenax*) used in this study were provided by the Kumamoto prefectural fisheries research center, the Kagoshima prefectural fisheries experiment station and the fisheries cooperative associations of Hokkaido and Tohoku area. All the algae were collected from January to June in 1996. Algae were cut in appropriate sizes and put into 10 mm diameter Teflon tubes and immediately measured by NMR spectroscopy at room temp. The NMR of samples stored at −20° or at room temp (22°) were measured two or four days after harvesting.

All of the reagents used were of analytical-reagent grade. A standard soln of 1000 $\mu g\ g^{-1}$ boron (Kanto Chemical Co., Tokyo, Japan) was diluted to the appropriate concns.

NMR measurement. ^{11}B NMR spectra data at 86.55 MHz were recorded on a GSX-270 (Jeol Ltd., Akishima, Japan). The NMR parameters used were: data point 8192, frequency range 20 kHz, flip angle 90 degrees (16.0 μs), repetition time 0.235 s, and exponential broadening factor 15 Hz.

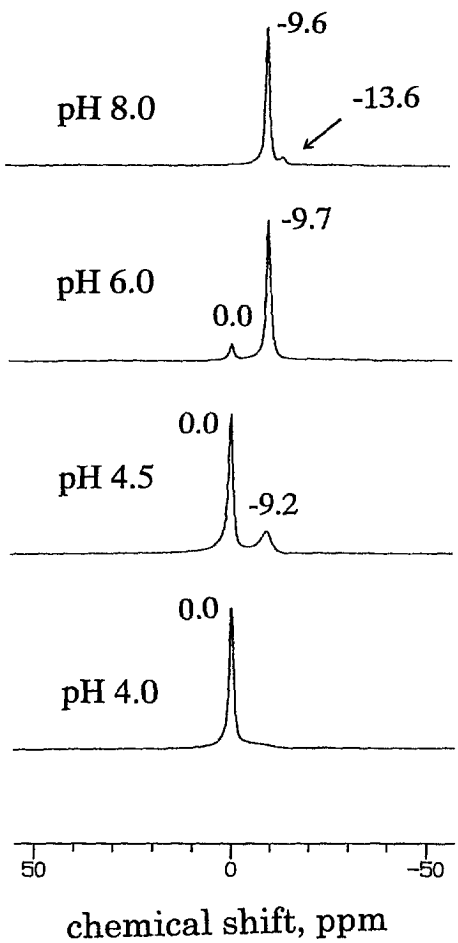

Fig. 3. ^{11}B NMR spectra of aqueous solutions of 0.2 M H_3BO_3 with 0.4 M mannitol as a function of the pH. The pH was adjusted with 1, 5 M NaOH or 1 M HCl. The spectrometer conditions were the same as those for the seaweed samples, except for scan numbers (1600).

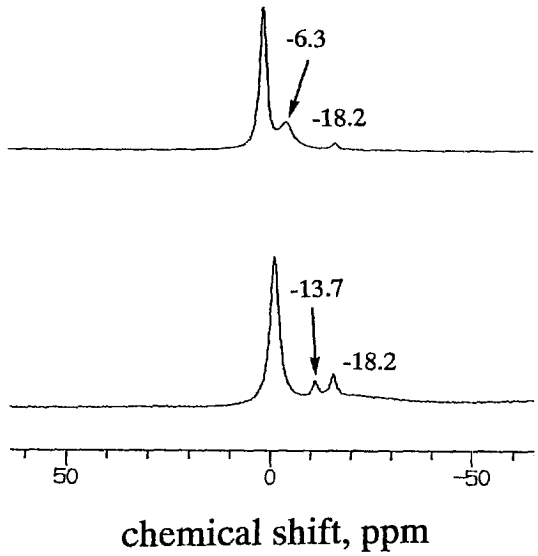

Fig. 4. ^{11}B NMR spectra of aqueous solutions of 80 mM H_3BO_3 with 5 g 100 ml^{-1} laminarin at pH 8.6 (below) and 460 nM H_3BO_3 with 6 g 150 ml^{-1} alginic acid at pH 7.1 (above). The pH was adjusted with 1, 5 M NaOH or 1 M HCl.

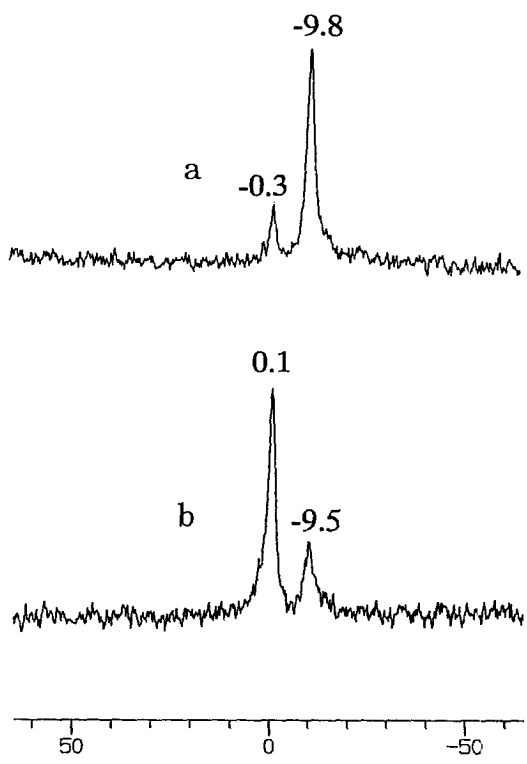

Fig. 5. *In vivo* ^{11}B NMR spectra of *Laminaria japonica*: a, two days after harvesting; b, four days after harvesting.

Tissue digestion for B estimation. The algae were lyophilized for 24 hr and homogenized in a Waring blender. Digestion units were polypropylene vessels equipped with inner vessels made of Teflon. All vessels were washed with 10 M HCl and rinsed with high-purity H_2O. The seaweed sample was directly weighed into a digestion vessel, and thoroughly mixed with HNO_3 (65%, 2 ml). After 10–15 min, H_2O_2 (30%, 1 ml) was added carefully. the vessels were capped and put into the microwave oven. A programme performed the heating steps (microwave power W, time min): 1 (200, 2), 2 (0, 2), 3 (250, 5) 4 (0, 3), 5 (300, 5). The vessels were cooled for 30 min in a H_2O bath before removal of the caps. The ICP measurement, the resulting digestion solns were diluted with high-purity H_2O.

ICP-AES conditions. The measurements were performed on a JICP-PS3000UV (Leeman Lab. Inc, Lowell, MA) with a cross-flow nebulizer. The conditions were: RF power 1.0 kW, gas flow 13 l min^{-1}, nebulizer gas pressure 40 psi, auxiliary gas flow 0 ml min^{-1}, sample aspiration rate 1.0 ml min^{-1}, wave length 249.678 nm.

Acknowledgements—We thank Dr Yasui, Mr T.

Suzuki and Ms K. Shindoh of this institute for helping to determine the boron contents in the seaweed by ICP-AES, and Dr H. Nasir, for helpful discussions. We thank also Mr S. Kowashi, Kumamoto Prefectural Fisheries Research Centre, for supplying seaweed. The Kagoshima Prefectural Fisheries Experiment Station and the Fisheries Cooperative Associations of Hachinohe, Miyako, Nemuro, Ochiishi and Taroucho are gratefully acknowledged for the kind gifts of seaweed.

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