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Tohoku Journal of Agricultural Research
Vol. 57 No. 1-2, November 2006
Printed in Japan

Polycavernosides Poisoning Caused by the Edible Red Alga *Gracilaria edulis* in Philippines

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(Received, April 4, 2006)

Summary

Outbreaks of seaweed poisonings are widely spread over the pacific area. Fatal glycosidic macrolides, polycavernosides (Yotus-Yamashita and Yasumoto *et al.*, 1993), and potent tumor promoters, aplysiatoxins (Nagai *et al.*, 1996), have been previously isolated from edible seaweed. During 2002-2003, three fatal poisoning incidents occurred resulting from ingestion of two edible red alga, *Acanthophora specifera* and *Gracilaria edulis*, in Philippines causing eight deaths among 36 patients. Analytical methods for polycavernosides and aplysiatoxins were first developed, and the causative toxin from *G. edulis*, collected during the second poisoning event on 2 December 2002, was then investigated. The semi-purified toxic fraction obtained from this alga based on mouse bioassay was applied to LC-diode array detection (LC-DAD) and LC/electrospray-MS (LC/ESI-MS) analyses. Both LC-DAD and LC/MS chromatograms of this fraction suggested the presence of polycavernoside A (PA) by comparison with the authentic PA.

Key words: polycavernosides, seaweed poisoning

Polycavernoside A (PA) (Fig. 1) and polycavernoside B (PB) were isolated as the causative toxins for fatal human intoxication resulting from ingestion of the red alga *Gracilaria edulis* (= *Polycavernosa tsudai*) that occurred in Guam in 1991 (Yotus-Yamashita and Yasumoto *et al.*, 1993). In this incident, thirteen people became ill, and three of them died. Other congeners, polycavernoside A2 (PA2), A3 (PA3), and B2 (PB2), were also isolated from the same alga collected in Guam in the next year (Yotus-Yamashita and Yasumoto *et al.*, 1995). The LD_{99} in mice (ip) of PA and PB was estimated to be 200–400 μg/kg for each, while that of PA2, PA3 and PB2 was not determined. Polycavernosides are characterized by macrolide structures possessing side chains containing a conjugated diene or triene, and *O*-methylated L-fucosyl-D-xylose. Total synthesis of PA has been achieved by three groups (Fujiwara and Murai *et al.*, 1998, Paquette *et al.*, 1999, White *et*

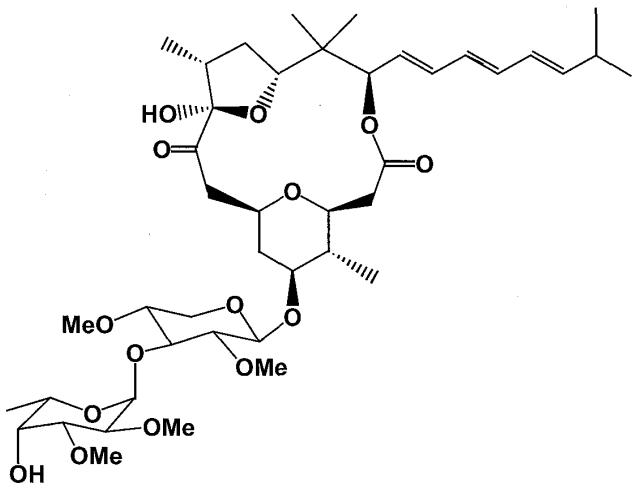

FIG. 1. The structure of polycavernoside A (PA)

al., 2001). According to the reported structure-activity relationship for the synthetic polycavernoside analogs, the aglycon with the conjugated polyene side chain is necessary for revelation of the toxicity to mice (Barriault, *et al.*, 1999). Pharmacology of polycavernosides has never been clarified yet because of limitation of sample supply. During 2002–2003, three fatal poisoning incidents occurred resulting from ingestion of two edible red alga, *Acanthophora specifera* and *Gracilaria edulis*, in Philippines causing eight deaths among 36 patients. Analytical methods for polycavernosides were first developed, and we identified PA as the toxic principle of this poisoning (Yotsu-Yamashita *et al.*, 2004).

Semi-purification of toxic fraction from G. edulis collected in Philippines

G. edulis (1.9 kg, wet weight) collected on 2nd December in 2002 at the beach in Luna, La Union, Philippines, was extracted with MeOH thrice, and the extract was filtered through cellulose filter. After partitioned between hexane and aqueous 80% MeOH (toxic) and between aqueous 40% MeOH and $CHCl_3$ (toxic), the residue of $CHCl_3$ fraction was chromatographed on a silica gel column and three reversed phase columns continuously. The toxic semi-purified fraction was obtained by mouse bioassay guided purification. Aliquots of this fraction were applied to LC-DAD, LC/ESI-MS and LC/ESI-MS/MS for analysis of polycavernosides.

Identification of polycavernoside A in the semi-purified toxic fraction by LC-DAD

On the LC-DAD chromatogram of the semi-purified toxic fraction from *G. edulis*, a peak possessing typical UV absorption for a conjugated triene at 259, 269, and 280 nm appeared at 9.51 min, indicating the presence of PA. The total amount of PA in the semi-purified toxic fraction from the *G. edulis* (0.65 kg, wet alga) was estimated to be 55 nmol (84 nmol/kg wet alga) by using the (remove)

calibration curve. Since the reported toxicity of PA to mice (LD_{99}, ip, 200–400 μg/kg) can be converted to 3–6 μg/MU for a 15 g body weight mouse, the above estimated amount of PA (55 nmol) was calculated at 8–15 MU, which roughly agreed with the experimentally determined toxicity to mice (10–20 MU).

Identification of polycavernoside A in the semi-purified toxic fraction by LC/ESI-MS

The mass chromatogram of the semi-purified toxic fraction obtained from the causative *G. edulis* monitored at m/z 847 in single ion monitoring (SIM) mode, clearly showed a peak corresponding to PA at 10.0 min. The total amount of PA in this fraction from 0.65 kg of wet *G. edulis* was estimated to be 47 nmol (72 nmol/kg wet alga) by using the calibration curve. The value was nearly compatible with that estimated by LC-DAD (84 nmol/kg wet alga). On the mass chromatogram detecting at m/z 821, a peak was shown at 8.00 min close to that of authentic PB2 (7.80 min). If we assign this peak to PB2, its peak pointed to roughly 4.6 nmol/kg wet alga. However, PB2 was below the detection limit by LC-DAD (0.06 nmol/kg alga) and by LC/ESI-MS/MS (less than 0.9 nmol/kg alga) as shown in LC-DAD and LC/ESI-MS/MS sections, respectively.

LC/ESI-MS/MS for polycavernosides

The characteristic fragmentation patterns were shown in polycavernosides by ESI-MS/MS and they were applied to LC/ESI-MS/MS. First, the solution containing authentic PA, PA2, PA3 and PB2 were applied to LC/ESI-MS/MS in SRM (single reaction monitoring) mode by monitoring at m/z 847–513, 833–513, 861–513 and 821–483 for PA, PA2, PA3 and PB2, respectively, as parent and daughter ion pairs. The semi-purified toxic fraction obtained from *G. edulis* containing approximately 780 pmol of PA quantified by LC-DAD was applied to LC/MS/MS in SRM mode by monitoring at m/z 847–513 and 847–673 for PA, and at m/z 821–483 and 821–633 for PB2. The peak corresponding to PA was clearly shown on the mass chromatograms detecting at m/z 847–513 and 847–673.

Conclusions

The amount of PA in the causative alga *G. edulis* was estimated as 84 nmol/kg and 72 nmol/kg, using the standard calibration curves for LC-DAD and for LC/ESI-MS in single ion monitoring (SIM) mode, respectively. Other polycavernoside congeners, A2, A3 and B2, and aplysiatoxin and debromoaplysiatoxin were less than the detection limit (2 nmol/kg alga, signal to noise ratio : 3) by LC/ESI-MS SIM analysis. In ESI-MS/MS, authentic polycavernosides showed the daughter ions corresponding to sequential loss of fucosylxylose residues. These fragmentations were applied to LC/ESI-MS/MS for polycavernosides in SRM

mode. On SRM mass chromatograms, the toxic fraction from the alga showed the peaks corresponding to PA, supporting the identification of PA as the cause of poisoning of *G. edulis* in Philippines.

Acknowledgements

The toxic seaweed collected in Philippines was provided by Prof. Takeshi Yasumoto (Japan Food Research Laboratories), Ms. Fe Farida A. Bajarias (Bureau of Fisheries and Aquatic Resources, Philippines), Ms. Mirriam A. Formelozsa (National Fisheries Research and Development Institute, Philippines), and Prof. Yasuo Fukuyo (University of Tokyo). This work was supported by grants-in-aid for Scientific Research on Priority Areas (no.17035011) from MEXT, (no. 18032014) from JSPS, and Nagase Science and Technology Foundation.

References

Barriault, L., Boulet, S.L., Fujiwara, K., Murai, A., Paquette, L.A. and Yotsu-Yamashita, M., *Bioorg. & Med. Chem. Lett.*, **9**, 2069-2072 (1999)

Fujiwara, K., Murai, A., Yotsu-Yamashita, M. and Yasumoto, T., *J. Am. Chem. Soc.*, **120**, 10770-10771 (1998)

Nagai, H., Yasumoto, T. and Hokama, Y., *Toxicon*, **34**, 753-761 (1996)

Paquette, L.A., Barriault, L. and Pissarnitski, D., *J. Am. Chem. Soc.*, **121**, 4542-4543 (1999)

Yotsu-Yamashita, M., Haddock, R.L. and Yasumoto, T., *J. Am. Chem. Soc.*, **115**, 1147-1148 (1993)

Yotsu-Yamashita, M., Seki, T., Paul, V.J., Naoki, H. and Yasumoto, T., *Tetrahedron Lett.*, **36**, 5563-5566 (1995)

Yotsu-Yamashita, M., Yasumoto, T., Yamada, S., Bajarias, F.F.A., Mirriam, A., Formelozsa, M.A. I, Romero, M.R. and Fukuyo, Y., *Chem. Res. Toxicol.*, **17**, 1265-1271 (2004)

White, J.D., Blakemore, P.R., Browder, C.C., Hong, J., Christopher, C.M., Nagornyy, P.A., Robarge, L.A. and Wardrop, D.J., *J. Am. Chem. Soc.*, **123**, 8593-8595 (2001)