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# Evaluation of α- and β-glucosidase inhibitory properties of macro-algae using intestinal extracts of marine snail, *Thais rudolphi* (Lamarck, 1822)

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Glucosidase-inhibitory activity of marine macro-algae, *Ulva fasciata* and *Hypnea musciformis* in different solvents was detected using enzyme-agar plate method. Eenzyme-agar plate containing α- or β-D-glucosidase was used in combination with substrate-agar plate containing *p*-nitro phenyl α- and β-D-glucopyranoside (PNPG). Discs impregnated with algal extracts placed on the enzyme agar, followed by pre-incubation up to 2.5 h at 25°C, resulted in the characteristic inhibitory circle around the inhibitor. Extracts of red algae, *H. musciformis* had higher glucosidase-inhibition than the extracts of *U. fasciata*. The intestine of *Thais rudolphi* contained both α- and β-glucosidase enzymes and their inhibition was comparable with the commercial enzymes, suggesting the possible alternate method for assessing the glucosidase-inhibitory activity of the marine macro-algae.

**Keywords**: Glucosidases, *Hypnea musciformis,* macro-algal inhibitors, rapid assay, *Thais rudolphi*, *Ulva fasciata*

Marine macro-algae represent one of the less explored sources of biologically active natural products with possible pharmaceutical applications[1]. The macro-algal polysaccharides contain therapeutic and bioactive agents for treating multiple diseases[2]. Novel compounds with antibiotic, anti-tumor, cytotoxic and anti-inflammatory activities have been isolated and elucidated[3]. Glucosidase inhibitors are potential bio-tools and highly useful for medical therapies, such as diabetes, obesity, hyperlipo-proteinemia, cancer, HIV and HBV[4]. α-D- and β-D-glucosidases are hydrolytic enzymes of α- and β-linkages of glucosides[5]. Inhibition of glucosidase is effective for diseases caused by high blood glucose level by controlling the kinetics of intestinal carbohydrate digestion[6].

Crude methanolic extract from *Pelvetia babingtonii* showed potent α-glucosidase inhibitory activity that could make it effective in suppressing postprandial hyperglycemia[7]. However, glucosidase inhibitions are rarely reported from macro-algae. One of the possible reasons is the lack of an efficient enzyme assay method suitable for macro-algae. The results of an attempt to find out the relative glucosidase inhibitory potential of two macro-algae, *Ulva fasciata* and *Hypnea musciformis* using a standard inhibitor, glucono-δ-lactone, are presented here. An alternate natural source for glucosidase enzyme was detected from the intestine of marine snail, *Thais rudolphi* and its efficacy in the inhibition potential of macro-algal extracts is also presented.

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## Materials and Methods

### Preparation of Extracts

Fresh macro-algae, *U. fasciata* was collected from the coastal rocks of Vizhinjam (Lat. 08° 22′ N; Long. 76° 59′ E), Southwest coast of India and *Hypnea musciformis* from Rameswaram/Mandapam regions (Lat. 09° 25′ N; Long. 79° 20′ E), Southeast coast of India during the post-monsoon months. Immediately after collection, the macro algae was washed in fresh seawater to remove the epiphytes, sand and other extraneous matter and dried under shade. The dried material was weighed and ground finely in a mechanical grinder.

The macro-algal crude extracts were prepared using methanol[8]. In this process, 500 g of finely powdered algal material was refluxed with methanol in a 5 L capacity round bottom flask. The extract was filtered and concentrated to recover the excess solvents in another distillation system. Finally, it was reduced to a thick viscous crude extract in a rotary vacuum evaporator (Buchi) at 40°C. Water extract was

prepared by homogenizing the fresh collection with required quantity of phosphate buffered saline (*p*H 7.4) and the extract was strained using a strainer. The quantity of water extract was measured further to make heat condensed extract by giving a heat treatment in a water bath at 40°C for 10 min. Fractionated part of macro-algal extracts along with their water and heat condensed extracts were used to analyze the glucosidase-inhibitory activity. Extracts of powdered *U. fasciata* using solvents methanol-acetone (M4), dichloromethane-acetone (DM2), fresh ethanol (EF), dichloromethane-methanol (M4) and ethyl acetate (ACO5) were also tested as fraction.

**Extraction of Glucosidase Enzyme from Marine Snail, *T. rudolphi***

In order to evaluate the glucosidase activity and compare the results with that of the macro-algal extracts, marine snails (*T. rudolphi)* intestinal extracts were used. *T. rudolphi* were collected from inter-tidal areas of Mulloor (Lat. 08° 22′ N; Long. 76° 59′ E), Southwest coast of India. Glucosidase from the snail was obtained from the intestinal wall (crop, stomach and intestine). Active snails were divided into two batches. The digestive tract of one batch (batch 1) was removed immediately after de-shelling and stored at –15°C. The other batch (batch 2) was kept in filtered seawater without feed. After 2 to 3 d of starvation, the snails in batch 2 were de-shelled to remove their digestive tract. The dissected contents were washed 4-times with 50 mL of 0.05 *M* sodium acetate buffer (*p*H 5.0). About 2 g (fresh wt) of the various sections of the washed gut wall was then homogenized manually using glass-tissue homogenizer in a minimum amount of acetate buffer (*p*H 5.0). The homogenates were then centrifuged at 5000 × g for 15 min at 4°C. One part of supernatant solution was stored at –15°C and the remaining part was mixed with twice their volume of cold acetone and left at 4°C for 30 min. The precipitate obtained after centrifugation at 5000× g for 15 min was separately dissolved in 0.05 *M* sodium acetate buffer (*p*H 5.0). After centrifugation, the supernatant solution was made up to 8.0 mL with the above buffer and portions of these solutions were used for enzyme assay. The extracted samples were divided into 4 sections like BA (before acetone-immediately killed), BAS (before acetone-starved), AA (after acetone-immediately killed) and AAS (after acetone-starved).

**Enzyme Assay**

The modified agar plate method[6] was used to screen extracts of *U. fasciata* and *H. musciformi* for their glucosidase inhibition activity. The α-D-glucosidase enzyme agar plate was prepared by adding 0.15 mL of 0.1 mg/mL α-glucosidase in 10 m*M* phosphate buffer (*p*H 7.0) onto an agar plate (1.5% agar in 20 mL of phosphate buffer). The substrate agar was prepared by dissolving 3.0% agar in 10 mL of 10 m*M* phosphate buffer (*p*H 7.0) containing the substrate 5.0 m*M* *p*-nitrophenyl α-D-glucopyranoside. The pre-determined concentra-tion of the extracts, such as 10% and 1%, of *U. fasciata* and *H. musciformis* along with known glucosidase inhibitor, glucono-δ-lactone, was impregnated onto each 6 mm sterile disc (Sterile Susceptibility Test Discs/Himedia). These sterile discs were placed inside the holes (6 mm) in the enzyme-agar plate and the bottom of each well was sealed with 1% agar. In each petri dish, 6 such wells were cut and placed with impregnated discs. The enzyme agar with the paper discs was pre-incubated at 25°C for 2.5 h and covered with the substrate agar. After incubation at 25°C for 30 min, the colourless zones around the paper discs against the yellow background were measured using Hi Antibiotic Zone scale-C (HiMedia). For β-D-glucosidase inhibition assay, the enzyme agar plate was made of 1.5% agar in 10 m*M* citrate buffer (*p*H 5.0, 20 mL) and 0.4 mg/mL β-D-glucosidase (0.15 mL), while the substrate agar plate was prepared by dissolving 3% agar in 10 m*M* citrate buffer (*p*H 5.0, 10 mL) containing 5 m*M* *p*-nitrophenyl β-D-glucopyranoside (PNPG). Subsequent procedures were the same as for the α-D-glucosidase inhibition assay. The enzyme agar covered with the substrate agar was left at 25°C for 30 min and then placed in a container saturated with ammonia vapour. The procedure is diagrammatically shown in Fig. 1.

**Non-denatured Gel Electrophoresis**

The non-denatured polyacrylamide gel (10 mL, 5%) was prepared as given by Thangaraj and Lipton[9]. For this, 30 µL each of BAS and BA sample, 10 µL of molecular weight marker (PMW-M, Genei) with equal volume of sample buffer were loaded. The native molecular weight of the glucosidase enzyme was calculated with comparison of marker proteins by a gel documentation software unit (Image Master 1D Elite* Version 3.01, Amershamacia Biotech, USA). The protein was estimated with bovine serum albumin as standard[10].

## Results

The crude methanol extracts of *H. musciformis* showed 96.40% inhibition activity against β-D-glucosidase, followed by the 91.66% inhibiton against α-D-glucosidase at 10% concentration. The water-soluble fractions exhibited 80% glucosidase inhibition, which was more or less same as that of its crude form (Table 1); whereas less activity was noted in the heat-condensed extract. The M4 fraction obtained by methanol-acetone combination of *U. fasciata* had 83.30% of comparable activity as that of the standard α-glucosidase inhibitor (Table 2). Almost similar α-glucosidase inhibitory activity was noted in extracts of dicholoro methane-acetic acid and fresh ethanol solvents. However, the activities were only 50% in the other two solvents as well as almost negligible in the water extracts of *U. fasciata*. Fresh ethanol extract of *U. fasciata* showed higher range of β-glucosidase inhibition, i.e. 87.33%, followed by 86.66% by its dichloromethane-acetic acid extract (Table 2). Comparison study also revealed that macro-algal extracts of *H. musciformis* exhibited higher range of glucosidase inhibition compared to *U. fasciata* extracts. Evaluation of *Thais*-extracted glucosidase inhibition by extracts of *H. musciformis* exhibited dominant inhibition of β-galactosidase as compared to its α-derivative, as noted from the similar results of commercial enzymes tested (Table 3). The protein estimation results indicated higher protein level (9.4 mg/mL) in BAS sample and lower in AAS sample (4.14 mg/mL) (Table 4). The BAS sample had more bands compared to BA sample on native PAGE. The BAS sample had the molecular weights of 96.7, 43.3, 29.4, 17.5 and 16.9 kDa proteins. Of these, the band with molecular weight 43.3 kDa could be the native enzyme glucosidase (Fig. 2).

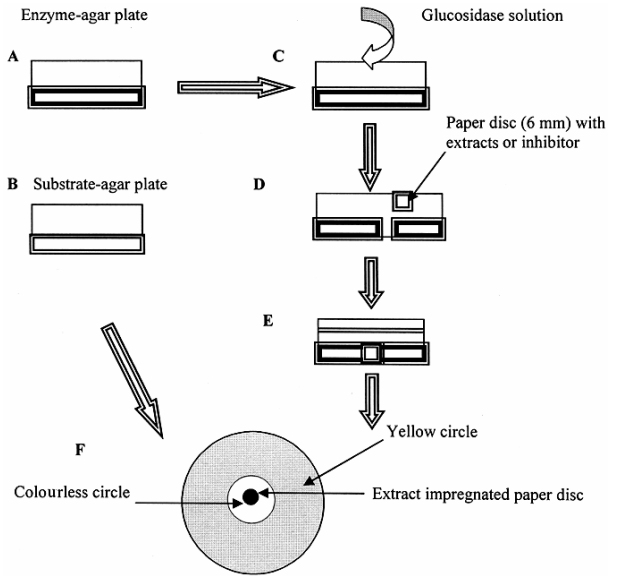


Fig. 1—Procedure of the agar plate assay

Table 1—Activity of water and methanol extracts of *H. musciformis*

| Enzyme | Concentration (%) | % inhibition of Methanol extract | Water extract | |
|---|---|---|---|---|
| | | | Pure | Heat condensed |
| α-Glucosidase | 10.0 | 91.66 | 80.00 | + |
| | 1.00 | 81.81 | + | + |
| β-Glucosidase | 10.0 | 96.40 | 76.80 | ++ |
| | 1.00 | 90.00 | 66.90 | + |

++: Medium inhibition (50.0%); +: Low inhibition (< 50.0%)

## Discussion

In order to screen potent novel marine natural products (MNPs), it is essential to develop reliable and rapid primary micro assay systems for the bioassay-guided purification and fractionation process[11]. Glucosidase activity has so far been measured by the indirect colorimetric method based

Table 2—Comparison of the activity of different solvent extracts of *U. fasciata*

| Enzyme | Concentration (%) | % inhibition of different purified organic extract | | | | | Water extract | |
|---|---|---|---|---|---|---|---|---|
| | | DM2 | M4 | EF | DM | ACO5 | Pure | Heat condensed |
| α-Glucosidase | 10.0 | 75.00 | 83.30 | 75.00 | 50.00 | 50.00 | + | + |
| | 1.00 | 72.72 | 72.72 | + | + | + | + | + |
| β-Glucosidase | 10.0 | 86.66 | 80.00 | 87.33 | 85.33 | 79.33 | 50.00 | + |
| | 1.00 | 84.61 | 78.21 | 84.52 | 76.80 | 68.56 | ++ | + |

DM2: Dichloromethane-acetic acid extract; M4: Methanol-acetone extract; EF: Fresh ethanol extract; M4: Dichloromethane-methanol extract; ACO5: Ethyl acetate extract; ++: medium inhibition (50%); +: Trace inhibition (< 50%).

Table 3—*Thais*-extracted glucosidase activity against *H. musciformis* extracts

| Substrate | Samples | | | |
|---|---|---|---|---|
| | BA | BAS | AA | AAS |
| *p*-Nitro phenyl α-D-glucopyranoside | ++ | +++ | + | + |
| *p*-Nitro phenyl β-D-glucopyranoside | ++ | +++ | + | + |

BA: Before acetone immediately killed; BAS: Before acetone-starved;
AA: After acetone-immediately killed; AAS: After acetone-starved;
+++: 60-80% inhibition; ++: 50.0% inhibition; +: < 50.0% inhibition

Table 4—Concentration of protein in different preparations

| Source | Protein (mg/mL) |
|---|---|
| BA | 8.6 |
| BAS | 9.4 |
| AA | 5.3 |
| AAS | 4.14 |

BA: Before acetone immediately killed;
BAS: Before acetone-starved;
AA: After acetone-immediately killed;
AAS: After acetone-starved

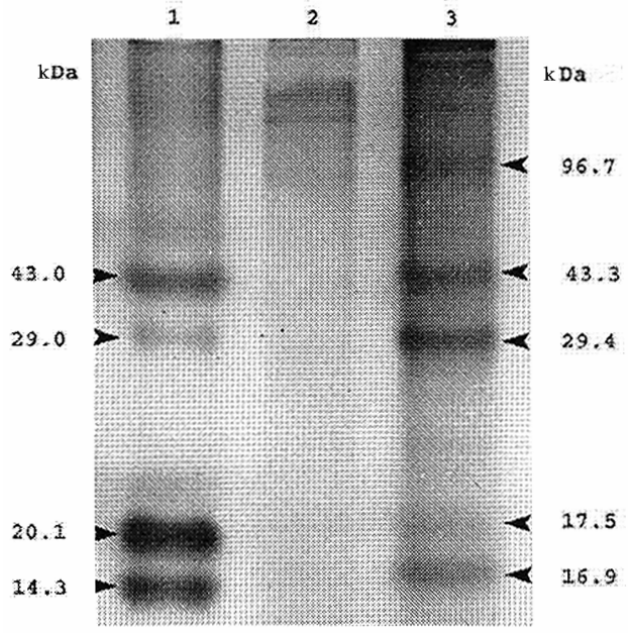


Fig. 2—Non-denatured PAGE (10 & 5%) of starved intestinal extract of *T. rudolphi*: Lane 1: Molecular weight marker (ovalbumin 43.1 kDa; carbonic anhydrase 29.0 kDa; soyabean trypsin inhibitor 21.1 kDa; lysozyme 14.3 kDa); Lane 2: BA sample; Lane 3: BAS sample.

on nitrophenol chromophore, a hydrolyzate of nitrophenyl glucosides, as a substrate[6]. The present agar plate method suggests a rapid visual judgment of both α- and β-glucosidase inhibition and thus is advantageous.

In the present study, most of the algal extracts showed more inhibitory properties against β-D-glucosidase compared to its α-derivative. Kurihara *et al*[6] reported glucosidase inhibitions against extracts of brown and red marine macro-algae, such as *Hizikia fusiforme, Sargassum thumbergii, Leathesia difformis, P. wrightii, Sevtosiphon lomenturius, Gloiopeltis furcata, Rhodoglossum japonicum, Corallina pilultfera* and *Calliarthron yessoense*. Results also revealed that crude methanolic extracts of *H. musciformis* exhibited stronger β-D-glucosidase inhibition property to a range of 96.40%, followed by 91.66% inhibition of α-D-glucosidase at 10.0% concentration. The differences in inhibition of α- and β-D-glucosidase may be due to the different polarities[6]. In the case of β-D-glucosidase, the stronger inhibitory activity shown by the water-soluble fractions was comparable with the earlier findings[6]. These glucosidase inhibitors change the sulphated polysaccharide groups of seaweeds[12,13]. Further, the lesser inhibition rate of heat condensed *H. musciformis* samples could be due to the reported diappearance of thermo-labile bioactive compounds at higher temperatures[14]. Moderate degree of inhibition (< 87%) by the fractionated extracts in comparison to the crude (96.40%) extract could be due to the modifications of active inhibitory compound during the fractionation process[15].

*Thais*-extracted-glucosidase enzyme activity against *H. musciformis* extracts could be considered a low cost and rapid method to detect the glucosidase from natural sources. An earlier finding of the presence of glucosides in *Achatina achatina*[16] along with the present findings suggests that snails provide rich source of glucosidase enzymes.

The result of non-denatured PAGE protein-banding pattern is comparable with earlier reported findings. Umezurike[15] reported a low molecular weight of 41 kDa glucosidase from the starved *A. achatina*. Udeugwu[16] also confirmed and characterized the same protein as 40 kDa from *A. achatina*. Thus, the starved marine snail *T. rudolphi* gut extract contained glucosidase with a possible native molecular weight of 43.3 kDa and warrants further studies to characterize these enzymes.

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## References

1 Smit A J, Medicinal and pharmaceutical uses of seaweed natural products: A review, *J Appl Phycol*, 16 (2004) 245-262.

2 Mayer A M S & Lehmann V K B, Marine pharmacology in 1998: Marine compounds with antibacterial, anticoagulant, antifungal, anti-inflammatory, anthelmintic, antiplatelet, antiprotozoal, and antiviral activities; with actions on the cardiovascular, endocrine, immune, and nervous systems; and other miscellaneous mechanisms of action, *Pharmacologist*, 42 (2000) 62-69.

3 Moore B S, Biosynthesis of marine natural products: Micro-organisms and macroalgae. *Nat Prod Rep*, 16 (1999) 653-674.

4 Mehta A, Zitzmann N, Rudd P M, Block T M & Dwek R A, α-Glucosidase inhibitors as potential broad based antiviral agents, *FEBS Lett*, 430 (1998) 17-22.

5 Chen H, Yan X, Lin W, Zheng L & Zhang W, A new method for screening α-glucosidase inhibitors and application to marine microorganisms, *Pharm Biol*, 42 (2004) 416-421.

6 Kurihara H, Sasaki M & Hatano M, A new screening method for glucosidase inhibitors and its application to algal extracts, *Fish Sci*, 60 (1994) 759-761.

7 Ohta T, Sasaki S, Oohori T, Yoshikawa S & Kurihara H, β-Glucosidase Inhibitory activity of a 70% methanol extract from ezoishige (*Pelvetia babingtonii* de Toni) and its effect on the elevation of blood glucose level in rats, *Biosci Biotechnol Biochem*, 66 (2002) 1552-1554.

8 Selvin J & Lipton A P, Biopotentials of *Ulva fasciata* and *Hypnea musciformis* collected from the peninsular coast of India, *J Mar Sci Technol*, 12 (2004) 1-6.

9 Thangaraj M & Lipton A P, Species-specific proteins in closely-related seahorses, *Curr Sci*, 86 (2004) 1645-1647.

10 Lowry O H, Rosenbrough N J, Farr A L & Randall R J, Protein measurement with the Folin phenol reagent, *J Biol Chem*, 193 (1951) 265-275.

11 Selvin J & Lipton A P, Development of a rapid 'mollusc foot adherence bioassay' for detecting potent antifouling bioactive compounds, *Curr Sci*, 83 (2002) 735-737.

12 Schaeffer D J & Krylov V S, Anti-HIV activity of extracts and compounds from algae and cyanobacteria, *Ecotoxicol Environ Safe*, 45 (2000) 208-227.

13 McCaffrey E J & Endan R, Antimicrobial activity of tropical and subtropical sponges, *Mar Biol*, 89 (1985) 1-8.

14 Chellaram C, Gnanambal K M E & Edward J K P, Antibacterial activity of the winged oyster *Pteria chinensis* (Pterioida: Pteridae), *Indian J Mar Sci*, 33 (2004) 369-372.

15 Umezurike G M, The β-glucosidase in the gut contents of the snail *Achatina achatina*, *Biochem J*, 157 (1976) 381-387.

16 Udeugwu F C, Studies on the properties of low molecular weight D-glucosidase in the gut contents of aestivating *Achatina acatina*. BSc Project, Department of Applied Biochemistry, Nnamdi Azikiwe University, Awka, Nigeria, 1987.