Purification by Chromatography and Properties of β-Glucosidase of Japanese Cycad

Purification by Chromatography and Properties of β-Glucosidase of Japanese Cycad

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Nishida[1] reported in his early studies on Japanese cycad, *Cycas revoluta* THUNB., that its seeds were a good source of β-glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21), which produced formaldehyde from a presumed toxic glycoside in the same seeds. One of the authors, Kobayashi[2], partially purified the enzyme in order to apply it for investigating the hydrolysis of cycasin. Cycasin is the predicted toxic component of the Japanese cycad, first isolated and elucidated as methylazoxymethyl-β-D-glucoside in this laboratory[3]. This paper describes about the further purification process of enzyme attained by subsequently progressed chromatographic techniques, and also its properties towards cycasin as the substrate.

Purification of many glycosidases was carried out by ion-exchange and gel-filtration procedures[4-7] which were applied in this study. The means employed for the purification of β-glucosidases of various origins were as follows; calcium phosphate-gel, DEAE-cellulose, and zone electrophoresis by Duerksen and Halvorson[8], DEAE-Sephadex by Helferich and Kleinschmidt[9], and DEAE-cellulose and Biogel P-150 by Rudick and Elbein[10]. The authors also attempted to apply the recent methods of affinity chromatography for the cycad enzyme, and the results are to be reported elsewhere.

Materials and Methods

Materials

The seeds of cyacd were collected on the campus of Faculty of Fisheries, Kagoshima University in May, 1973. Those harvested at Kasari, Amami-Ohshima, Kagoshima in July, 1975 were also used partly.

DEAE-cellulose of Serva Co., CM-cellulose of Brown Co., Sephadex G-200 of Pharmacia Co., and other reagents of the purest grade were available from commercial sources. Cycasin was isolated from the cycad seeds in this laboratory. ONPG*[3] as the substrate was synthesized according to the method for the p-isomer of Glaser and Walwek[11].

Assay of Enzyme Activity

Basal conditions for the assay of enzyme activity towards ONPG as the substrate were as follows; 0.5 m*l* of 24 mM solution of ONPG and 1.0 m*l* of 0.2 M acetate buffer, pH 5.0,

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*3 o-nitrophenyl-β-D-glucoside

were preincubated at 30° for 5 min, and then were added with 0.5 m*l* of appropriately diluted enzyme solution. Samples of 0.2 m*l* each from the reaction mixture were withdrawn into 4.8 m*l* of 0.1 M solution of sodium carbonate at 0 time and 5 min intervals. The amount of o-nitrophenol freed from the substrate was determined from the optical density at 420 nm. The initial reaction velocity was obtained as the μmole amount of liberated o-nitrophenol per 1.0 m*l* of the reaction mixture per min. The reaction velocity constant of first-order, k, from the ratio of hydrolysis, and the unit of enzyme activity, u, from an equation of $u = k/\log 2$ were calculated.

When cycasin was used as the substrate, the sample solution from the similarily prepared reaction mixture as above was heated in a bath of 120° for 10 min in order to stop the enzymatic reaction. The amounts of residual cycasin and liberated glucose were determined by the gas chromatographic analysis of Kobayashi *et al.*[12] The initial reaction velocity was defined as the μmole amount of produced glucose per 1.0 m*l* of the reaction mixture per min.

Other Assays

Protein was determined by the modified method of Lowry[13]. The calibration curve was made upon the crude enzyme preparation on which the protein-nitrogen was analyzed by micro-Kjeldahl method. Protein in each fraction of the column chromatography was represented as the absorption at 280 nm. The photometers employed were 3 types of Hitachi 101, EPU-2A, and EPS-3T. A gas chromatograph of Yanagimoto G-8 equipped with a digital integrator, GPI-200, was used.

Results and Discussion

Preparation of Crude Enzyme

Kernels of the hulled seeds of cycad, 5 kg, were homogenized in a mixer together with 7.5 *l* of cold water, and stood overnight. The supernatant (Fraction I) was decanted from precipitated starch.

Dilute acetic acid was added to make pH of the solution as 4.4, and the precipitated voluminous protein was removed by centrifugation and filtration through a layer of Celite-545. The filtrate, 7.27 *l*, was made as Fraction II. This procedure was very effective[2] for the removal of viscous protein having no activity of β-glucosidase.

Solid ammonium sulfate was added to make 0.4 to 0.6 saturation of the salt, and the precipitate collected by centrifugation was dissolved in water and dialyzed against water in Visking cellophane tubes. A small amount of protein with no enzyme activity precipitated during the dialysis. The supernatant solution, 260 m*l*, was made as the crude enzyme preparation (Fraction III), which could be stored at 4° without any significant loss of activity for at least 2 months.

Column Chromatography

DEAE-cellulose preliminarily equilibrated with 0.01 M phosphate buffer, pH 6.0, was packed in a column of ϕ 2.4 × 49 cm. Fifty m*l* of the Fraction III was applied on the column, which was eluted with the same buffer and secondly with the one containing 0.5 M sodium chloride. As shown in Fig. 1, the protein fractions which passed through the column exhibited the enzyme activity, while those eluted with the salt-containing buffer were without any activity. The former, Fraction IV, was applied on a CM-cellulose-cloumn of ϕ 2.6 × 87 cm buffered with 0.01 M phosphate buffer, pH 6.0. The result of chromatography was as

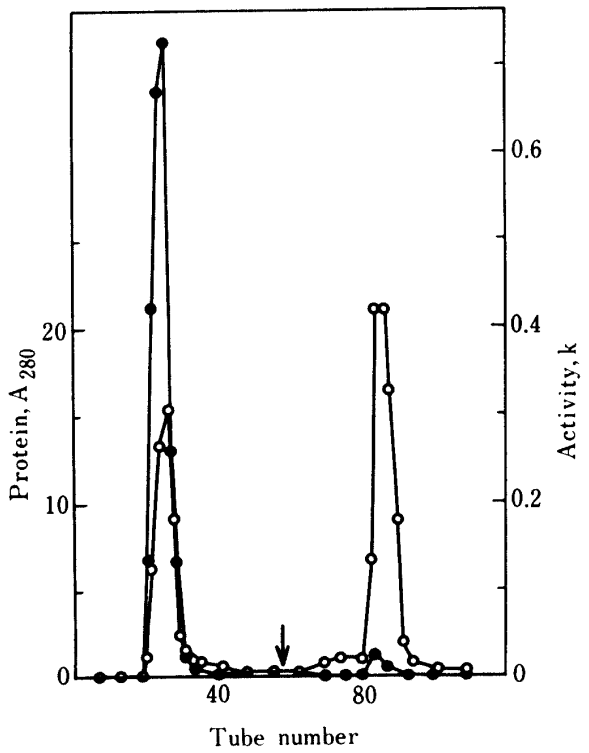

Fig. 1. Chromatography of cycad β-glucosidase on DEAE-cellulose. Sample: Fraction III. Column: ϕ 2.4 × 49 cm. Eluting buffer: 0.01 M phosphate buffer, pH 6.0, and 0.5 M NaCl in the same buffer. The arrow indicates the point where the application of salt-containing buffer was started. Tube size: 9.3 m*l*. Open circle: protein. Closed circle: activity.

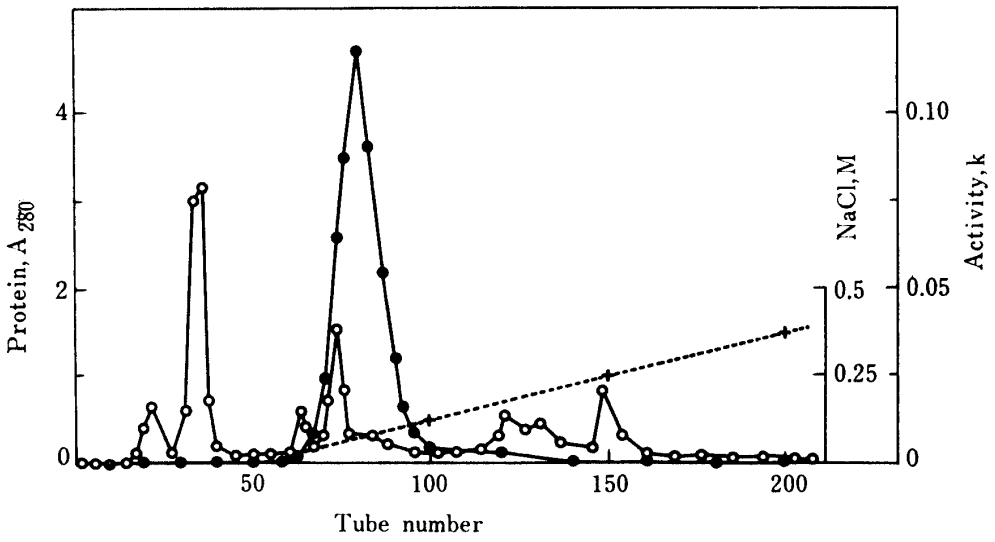

Fig. 2. Chromatography of cycad β-glucosidase on CM-cellulose. Sample: Fraction IV, 50 m*l*. Column: ϕ2.6 × 87 cm. Eluting buffer: linear gradient between 2.0 *l* each of 0.01 M phosphate buffer, pH 6.0, and 0.5 M NaCl in the same buffer. Tube size: 19.7 m*l*. Open circle: protein. Closed circle: activity. Cross: concentration of NaCl.

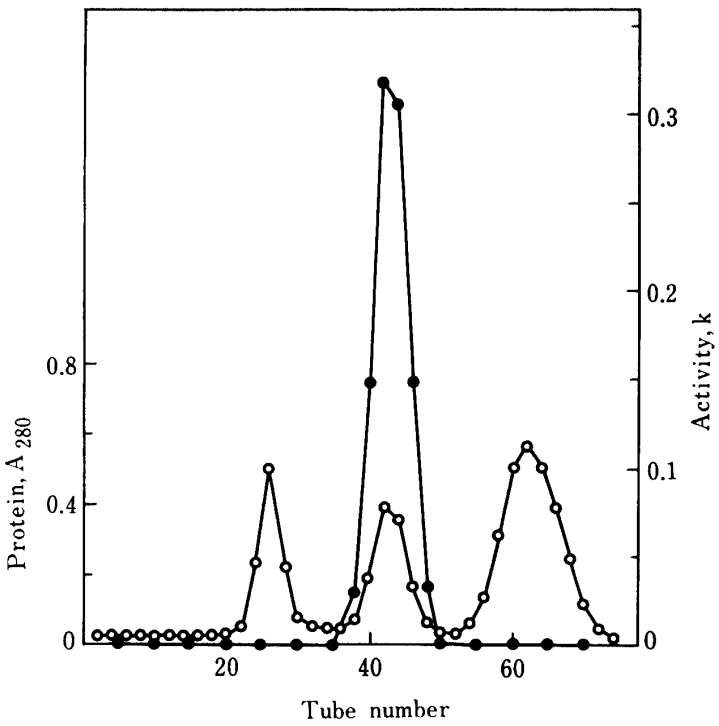

Fig. 3. Gel-filtration of cycad β-glucosidase through Sephadex G-200. Sample: Fraction V, 135 m*l*. Column: φ 2.0 × 93 cm. Eluting buffer: 0.1 M NaCl in 0.01 M phosphate buffer, pH 6.0. Tube size: 3.0 m*l*. Open circle: protein. Closed circle: activity.

Table 1. Purification of cycad β-glucosidase

Fraction		Volume	Total protein	Total activity	Specific activity	Yield	Purification
I	Initial extract	8,110 m*l*	244.9 g	2,866.4 u	0.012 u/mg	100 %	1
II	Filtrate after pH 4.4	7,270	21.7	2,390.8	0.110	83.4	9.4
III	$Am_2 SO_4$, 0.4 to 0.6 saturation	260	10.3	1,675.6	0.163	58.5	13.9
IV	DEAE-cellulose chromatography*1	65	626.6 mg	229.7	0.367	41.7	31.3
V	CM-cellulose chromatography	270	87.2	160.0	1.835	27.9	156.8
VI	Sephadex G-200 gel-filtration*2	18	2.4	31.5	13.237	10.0	1,131.4

* 1 A part of Fraction III, 50 m*l*, was applied to DEAE-cellulose chromatography.
* 2 The half of Fraction V, 135 m*l*, was applied to gel-filtration through Sephadex G-200.

shown in Fig. 2, and the enzyme activity was not found in the first eluate with 1 *l* of the same buffer but was held on the column. Elution of the enzyme was attained by gradient elevation of the ionic strength obtained by mixing 2 *l* each of the buffer and the one containing 0.5 M sodium chloride. The activity was found in the fractions eluted at the salt-concentration of about 0.1 M (Fraction V).

For the gel-filtration, one half of the Fraction V, 135 m*l*, was concentrated to 1.5 m*l* by the use of collodion bags, and was applied on a column of Sephadex G-200 (ϕ 2.0 × 93cm). Phosphate buffer of 0.01 M, pH 6.0, containing 0.1 M sodium chloride was used for the buffering and eluting of the column. As shown in Fig. 3, protein was eluted into clearly separated three peaks, and the center one of those peaks retained the enzyme activity. The last purified enzyme preparation obtained here, Fraction VI, showed an about 1,100-fold specific activity compared with the initial extract and its yield was 10 %.

The above-mentioned procedures of pruification were summarized in Table 1.

Properties of the Purified Enzyme Preparation

The enzyme of Fraction VI was used in the following experiments.

1. General Properties

According to the usual method[14] of disc electrophoresis, the enzyme was migrated on polyacrylamide-gels for pH 4.0 and 9.4. A single band of protein with enzyme activity was obtained in both experiments, and the preparation was proved to be homogenous by this method.

The ultra-violet absorpiton sepctrum was measured on an enzyme solution in 0.01 M phosphate buffer, pH 6.0, containing 0.1 M sodium chloride, and a characteristic spectrum of protein of λ_{max} 279 and λ_{min} 245 nm was observed.

Relationship between the amount of enzyme and the initial reaction velocity was examined on ONPG as the substrate, and was shown as in Fig. 4 to be a linear one upto the enzyme amount of 6.8 μ g.

Thermostability of the enzyme was surveyed by incubating the enzyme solution in 0.2 M acetate buffer, pH 5.0, at various temperatures for 10 min. The activity to ONPG assayed at 30° was, as shown in Fig. 5, lowered by above 40°-treatment, and lost completely at 80°.

Effects of various cations and few inhibitors upon the activity of enzyme were shown in

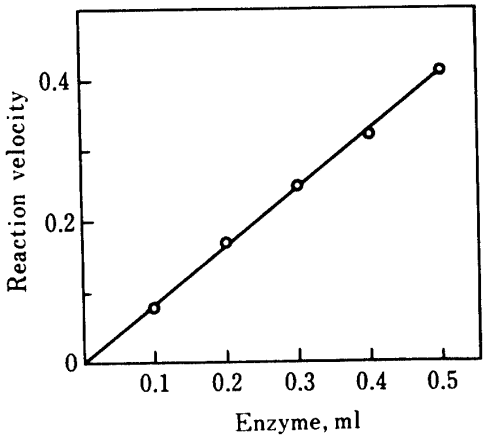

Fig. 4. Relationship between amount of enzyme and reaction velocity. Substrate: ONPG, 15 μ moles/m*l*. Enzyme: diluted solution of Fraction VI, 13.7 u/m*l*. Buffer: 0.2 M acetate, pH 5.0. Temperature: 30°. Reaction velocity: liberated o-nitrophenol, μmoles/m*l*/min.

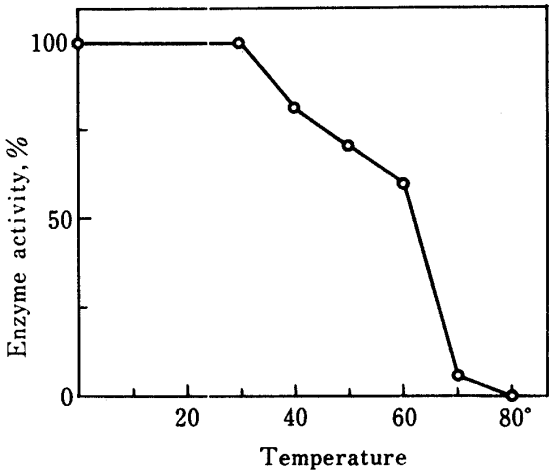

Fig. 5. Thermostability of enzyme activity. Substrate: ONPG, 6 μ moles/m*l*. Enzyme: diluted solution of Fraction VI, 0.04 u/m*l*. Buffer: 0.2 M acetate, pH 5.0.

Table 2. Effect of some metallic ions and inhibitors upon activity of cycad β-glucosidase

1 mM of	Relative activity
$HgCl_2$	101 %
$CaCl_2$	104
$MnCl_2$	120
$MgCl_2$	114
$ZnCl_2$	94
$CuSO_4$	105
$FeSO_4$	119
$Pb(CH_3COO)_2$	76
DFP*1	95
EDTA-2Na*2	106

Substrate: ONPG, 6 μmoles/ml. Enzyme: diluted solution of Fraction VI, 0.06 u/ml. Buffer: 0.2 M acetate, pH 5.0. *1 diispropyl fluorophosphate. *2 disodium ethylenediamine tetra-acetate.

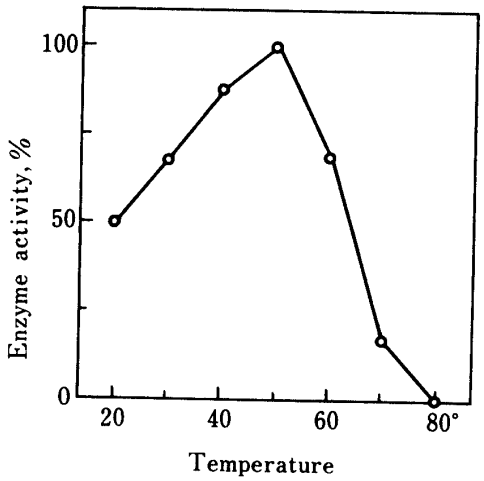

Fig. 6. Dependence of enzyme activity upon temperature. Substrate: ONPG, 6 μ moles/ml. Enzyme: diluted solution of Fraction VI, 0.04 u/ml. Buffer: 0.2 M acetate, pH 5.0.

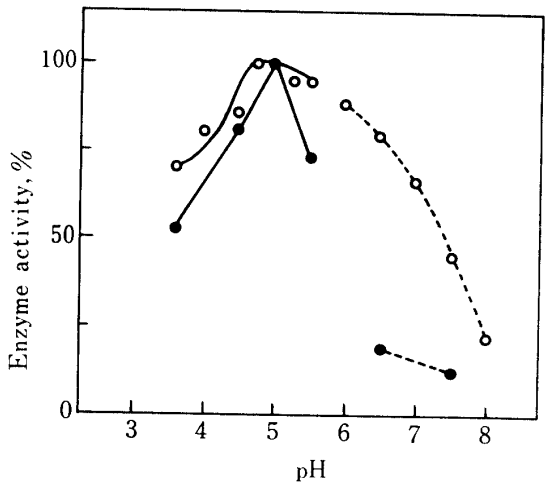

Fig. 7. Dependence of enzyme activity upon pH. Substrate: open circle, ONPG, 15 μmoles/ml; closed circle, cycasin, 10 μmoles/ml. Enzyme: diluted solution of Fraction VI, 0.04 u/ml. Buffer: solid line, 0.2 M acetate; dotted line, 0.2 M phosphate. Temperature: 30°.

Table 2 where ONPG was used as the substrate.

The optimum temperature for the enzyme activity to ONPG was 50° as shown in Fig. 6. The optimum pH was 5.0 both to ONPG and to cycasin as indicated in Fig. 7.

2. Kinetics

The Michaelis constant, Km, the maximum reaction velocity, Vmax, and the activation energy were investigated by the usual procedures upon the two substrates, ONPG and cycasin. From the Lineweaver-Burk's plot shown in Fig. 8, values obtained were Km = 2.8 and 10.0 mM each for ONPG and cycasin, and Vmax = 0.20 and 0.13 μ moles/min/ml each.

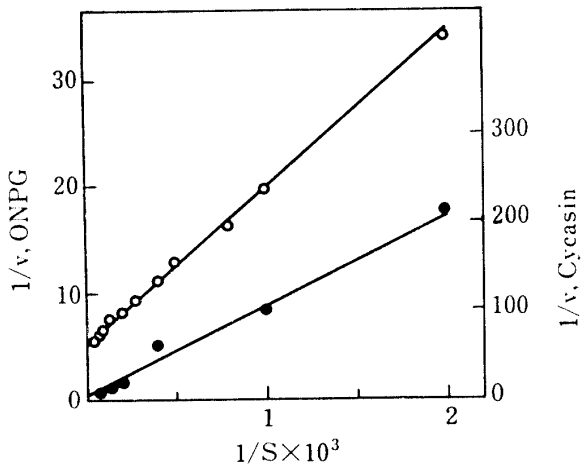

Fig. 8. Relationship between substrate concentration and reaction velocity.
Substrate: open circle, ONPG; solid circle, cycasin. Enzyme: diluted solution of Fraction VI, 0.11u/m*l*. Buffer: 0.2 M acetate, pH 5.0. Temperature: 30°.

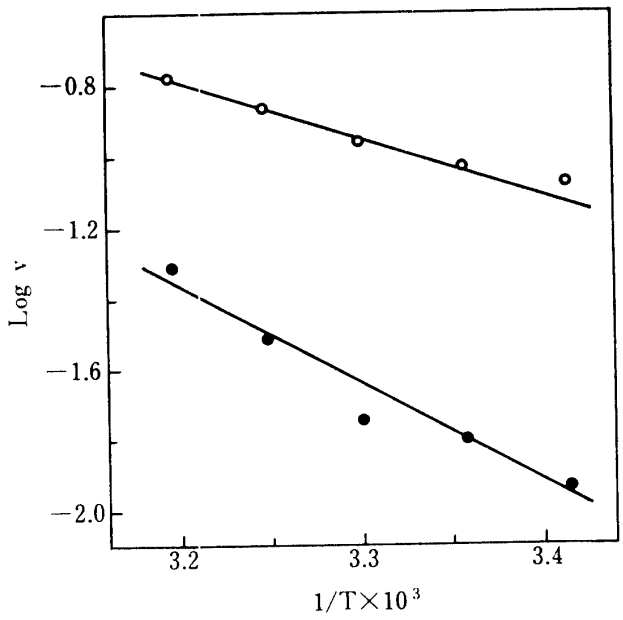

Fig. 9. Arrhenius plot of hydrolysis of ONPG and cycasin.
Substrate: open circle, ONPG, 15 μmoles/m*l*; solid circle, cycasin, 15 μmoles/m*l*. Enzyme: diluted solution of Fraction VI, 0.11 u/m*l* for ONPG and 0.19 u/m*l* for cycasin. Buffer: 0.2 M acetate, pH 5.0. Temperature: 30°.

The activation energy calculated from the Arrhenius' plot of Fig. 9 was 7.2 and 12.0 kcal each for ONPG and cycasin. The values of kinetics obtained here for ONPG were closely similar to the previous report[2], and those for cycasin, the natural substrate, were higher than for synthetic ONPG, which was shown to be a better substrate.

Analysis of the liberated glucose by reductometry, for example the Somogyi-Nelson's method, was not applicable in this case due to the labileness of the remained cycasin towards the alkalinity of the reagent. Although gas chromatography was useful for the simultaneous determination of glucose and cyasin, some difficulties were met on the sampling from the reaction mixture at a regular interval. After trials for stopping the enzymatic reaction by heating, acidification, or deproteinizing with trichloroacetic acid etc., procedures other than heating were found to bring troubles such as deposit of salts in the derivating trimethylsilyl compounds for gas chromatography. Heating was, however, not necessarily the best way especially in determining the activation energy on which temperature would have eminent influences. Determination of the reaction velocity for cycasin might have some problems to be examined further.

Summary

1. β-Glucosidase of the Japanese cycad was extracted with water from the homogenates of the seeds, acidified with acetic acid to pH 4.4, and fractionarily precipitated with ammonium sulfate, and a stable crude enzyme preparation was obtained. It was

further purified by DEAE-cellulose-, CM-cellulose-, and Sephadex G-200-column chromatographies. The purification was 1,100-fold of the initial extract and the yield was 10 %.

2. The purified preparation was homogenous in the disc electrophoresis on polyacrylamide-gel. Some properties and the kinetics of the preparation were studied for ONPG and cycasin as the substrates. Both the Michaelis constant and the activation energy were higher for cycasin, the natural substrate of the enzyme, than for synthetic ONPG.

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