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Isolation and Partial Characterization of Heterophyllin, a New Lectin from *Artocarpus heterophyllus* Seeds

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SUMMARY. Four lectins present in the seeds of jackfruit (*Artocarpus heterophyllus*, Lamk.) were isolated by employing solubility criteria and by guar gum, chitin and agarose-D-mannose affinity chromatography. One of the two lectins that bound to guar gum behaved as an albumin (AII) and was identified as the well known jacalin lectin by its sugar specificity, electrophoretic mobility and N-terminal amino acid sequence. The other guar gum binding lectin (GII) had the same above properties but was distinguished by its globulin nature. An albumin lectin (AIMII) which did not bind to guar gum was isolated due to its association with agarose-D-mannose and was identified as the lectin artocarpin. A newly identified lectin was isolated from the globulin fraction through its association with chitin. Chitin binding of the lectin was competed by N-acetyl-D-glucosamine. The new lectin designated heterophyllin, with a pI value of 6.5, contained three subunits of molecular weight 31.4, 18.7 and 16.3 kDa as estimated by SDS-PAGE.

RESUMEN. "Aislamiento y Caracterización Parcial de Heterofilina, una Nueva Lectina de Semillas de *Artocarpus heterophyllus*". Fueron aisladas cuatro (4) lectinas presentes en las semillas de *Artocarpus heterophyllus* Lamk. ("jaca") mediante el uso de criterios de solubilidad y varias cromatografias de afinidad: goma de guar, quitina y agarosa-D-manosa. De las dos lectinas que tuvieron afinidad a la goma de guar, una se comportó como albúmina (AII) y fue identificada como la bien conocida lectina "jacalina" por su especificidad al azúcar, movilidad electroforética y secuencia de aminoácidos en el extremo N-terminal. La otra lectina (GII) que se une a la goma de guar tiene las mismas propiedades anteriormente mencionadas, a excepción de su naturaleza globulínica. La lectina albumínica (AIMII), la cual no se unió a la goma de guar, fue aislada gracias a su asociación con agarosa-D-manosa y fue identificada como la lectina artocarpina previamente descripta. Una nueva lectina fue aislada de la fracción globulínica a través de su asociación con quitina. La unión de esta lectina com a quitina fue inhibida por N-acetil-D-glucosamina. La nueva lectina, designada heterofilina, con un pI de 6,5, contiene tres subunidades de pesos moleculares 31,4, 18,7 y 16,3 kDa, según se ha determinado por SDS-PAGE.

INTRODUCTION

Seeds of jackfruit (*Artocarpus heterophyllus*, Lamk. or *Artocarpus integrifolia*, Linn., Moraceae) are known since the late seventies to contain a lectin, namely jacalin, which is a glycoprotein Galβ (1→3) GalNAc binding lectin [1, 2]. Lately, a mannose binding lectin has been described from these seeds [3, 4] and more recent studies have pointed out to the existence of multiple jacalin isolectins [5]. Apart from crystallographic data [6] not much information on molecular structures and biological properties is known for these lectins.

Immunologists have been interested in jacalin for several reasons: it has been reported that jackfruit seed crude extracts show mitogenic properties towards human lymphocytes [7], increase the production of interferon-gamma by human-T-cells [8] and produce an enhanced IgE response [9] in mice initially fed and subsequently immunized with the same extract. Lately, jacalin has been shown to bind to IgA and to IgD [10, 11] and to inhibit C1 inhibitor [12]. More recently, jacalin has been reported to induce CD4-T lymphocyte proliferation with no effect on CD8-T lymphocytes in humans [13]. Furthermore, jacalin has been shown to inhibit human immunodeficiency virus [14].

KEY WORDS: Albumin, *Artocarpus heterophyllus*, Globulin, Heterophyllin, Jacalin, Lectins.
PALABRAS CLAVE: Albúmina, *Artocarpus heterophyllus*, Globulina, Heterofilina, Jacalina, Lectina.

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Although it is acknowledged that the immunological applications of jacalin will multiply [15], somewhat contradictory results concerning the proliferative response of human lymphocytes induced by either jackfruit crude extract [4] or another lectin, also present in the seeds [4] have been reported. These controversies might be explained by the existence of different isoforms of lectin(s) present in these seeds. Young *et al.* [16] had already shown that the molecule of jacalin is formed by two polypeptide chains known as α and β subunits with 113 and 20 aminoacid residues, respectively. A more detailed analysis of these aminoacid sequences disclosed that both α and β subunits possess variations in their structures, corroborating then the idea of the presence of multiple isoforms for jacalin. Lately, Young and Czapla [17] isolated and characterized four jacalin cDNA clones emphasizing the existence of multiple jacalin isolectins in jackfruit seeds.

After analysis of different protein fractions from *A. heterophyllus* seeds based on hemagglutinating activities and sugar inhibition of these fractions we could identify a peculiar behaviour of one of the analyzed fractions when compared to the already known lectins, artocarpin and jacalin. This finding prompted us to do a more through analysis of the lectin content of jackfruit seeds.

MATERIALS AND METHODS

Seeds

Seeds of *Artocarpus heterophyllus,* Lamk. (jackfruit, variety mole) were collected in Riacho da Guia (state of Bahia, Brazil).

Protein purification

Extraction. The seeds were decorticated, washed with acetone, dried out and ground to a fine powder in a blade mill. The resulting meal was sifted through a 60 mesh screen and proteins were extracted in PBS (25 mM phosphate buffer, 150 mM NaCl, pH 7.4, 1:10 ratio w/v) for 3 hours at 4 °C under constant stirring. The suspension was centrifuged at 10,000 x g and the supernatant (CE) was dialyzed for 48 h against distilled water. After dialysis the suspension was centrifuged at 10,000 x g for 30 min and the clear supernatant, labeled albumin fraction (AF) was kept at 4 °C until use. The pellet was dialyzed against water (three times) and centrifuged (10,000 x g, 30 min.) for the complete separation of the globulin fraction (GF) free of albumin contamination.

Guar gum chromatography. Guar gum which is a linear chain of D-mannose units with attached α-galactopyranoside units linked α (1–6) as single unit side chains, was used to prepare a column (2.6 cm diameter and 9.3 cm height) according to Fugita *et al.* [18]. The globulin fraction was loaded on the column which was equilibrated with 0.2 M glycine (pH 8.0) and washed with the same buffer until the absorbance of the effluent at 280 nm was less than 0.01. The fraction eluted with the equilibrium buffer was collected and named GI. The bound peak, eluted with 0.1 N HCl and neutralized with NaOH, was also collected and named GII. For the albumin fraction (AF), the column was equilibrated with PBS (pH 7.4), washed with the same buffer and unbound proteins were collected and named AI. The bound proteins were eluted with 0.1 N HCl and labeled AII. All active fractions were pooled, dialyzed against water for 48 h and freeze dried.

Chitin chromatography. Chitin used for affinity chromatography was prepared according to Hackman and Goldberg [19] as modified by Leopoldo *et al.* [20]. The fraction which did not bind to the guar gum column (GI, 10 mg) was dissolved in 5 ml of 50 mM phosphate buffer (pH 7.4) and centrifuged at 3,000 x g for 5 min. The clear solution was loaded on a chitin column (2.7 cm diameter and 4.6 cm height) which was equilibrated with the same buffer. After washing with the equilibrium buffer, the unbound fraction was collected and named GIQI. Bound proteins were eluted with 0.1 N HCl and after collection were neutralized with NaOH (GIQII). GIQI fraction was dialyzed against distilled water and freeze dried while GIQII was first dialyzed against 0.1 N acetic acid, followed by dialysis against distilled water and freeze dried.

Agarose-D-mannose chromatography. The unbound fraction (AI) from the chromatographic step in guar gum (4 mg in 2 ml) was applied to a column of agarose-D-mannose previously equilibrated with PBS (pH 7.0). The column was rinsed with the same buffer until the complete elution of the unbound proteins (AIMI) was obtained. Active fractions (AIMII) were eluted with 0.1 N HCl and after neutralization with NaOH were pooled, dialyzed against distilled water and lyophilized (AIMII).

Protein determinations

Protein content was estimated by the dye-binding method of Bradford [21] or by U.V. absorbance at 280 nm.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Electrophoresis was carried out according to the method developed by Laemmli [22] on 12% polyacrylamide slab gels.

pI determination

The isoelectric point of the GIQII fraction was measured on a native 7% polyacrylamide gel containing ampholytes to give a pH range of 3.5-10. Electrophoresis was running during 90 min at 200 V and for 90 min more at 400 V. The proteins were stained by Coomassie brilliant blue R-250 and the pH gradient was measured by slicing the gel into 5 mm sections, extracting them in 1.0 ml H_2O and measuring the pH values by using a glass electrode.

Hemagglutinating activity and its inhibition by sugars

The hemagglutination test utilizing rabbit red blood cells was done by the method of Moreira & Perrone [23]. Samples were serially diluted with 0.15 M NaCl to a final volume of 150 µl to which 50 µl of an 8% suspension of red blood cells were added. After incubation at 37 °C for 30 min hemmagglutination was observed and readings were taken in an optical microscope. The hemagglutination titer is defined as the maximal dilution of the sample still capable of giving 50% of erythrocyte agglutination. Additionally, a hemagglutinating unit (UH) is defined as the quantity of sample (µg of protein) capable of giving a hemmaglutination of 50% of the blood cells [24]. Inhibition of the hemagglutination activity by several sugars (N-acetyl-D-glucosamine, N-acetyl-D-galactosamine, α-methyl-mannoside, galactose, fructose and raffinose) was done by incubating the appropriately diluted (4 -500 mM) sugar solutions.

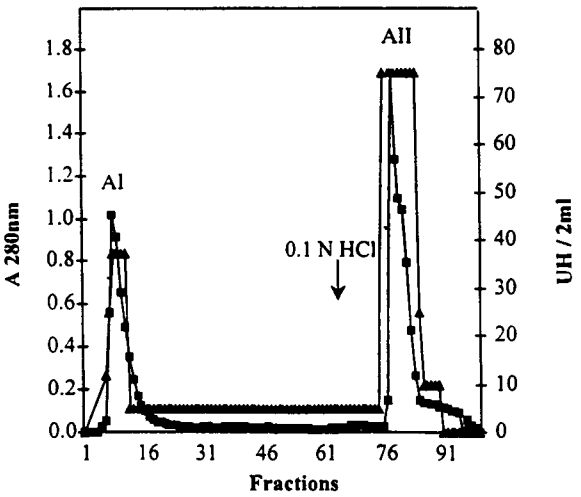

Figure 1. Guar gum affinity chromatography of the albumin fraction (AF) of A. heterophyllus (30 mg). A column of guar gum was prepared as it is indicated in Materials and Methods. It was equilibrated with PBS (0.05 M sodium phosphate, pH 7.4 and 0.15 M NaCl). The elution was done with the same solution followed by desorption of the adsorbed proteins by 0.1 N HCl. Fractions of 3.0 ml were collected. ■ Absorbance at 280 nm; ▲: Hemagglutinating titer.

N-terminal sequences of the protein

N-terminal sequences were determined in an automatic sequencer PSQ-10 Model (SHIMADZU). Sequence comparisons were done by using the Blast-algorithm and the NIH sequencing Bank according to Devreux *et al.* [25].

RESULTS AND DISCUSSION

Two protein fractions from seeds of the jackfruit were distinguished on the basis of their water solubility: an albumin fraction (water soluble) and the globulin fraction (water insoluble). These protein pools were further fractionated by affinity chromatography on guar gum, chitin and agarose-D-mannose columns. From the albumin fraction we obtained two fractions with lectin activity: an unbound fraction AI, and fraction AII which was bound to the guar gum column (Fig. 1). Two others fractions were obtained from the globulin fraction: the unbound fraction GI and GII which was adsorbed to the column of guar gum (Fig. 2). The hemagglutinating activities of GI and GII, originating from the globulin fraction were always higher than those of fractions AI and AII (Table 1).

Fraction	1 UH*
CE	0.780
AF	0.390
GF	0.620
G I	0.390
G II	0.104
A I	3.900
A II	2.020
GQ II	1.500
AIMI	25.000
AIMII	2.500

Table 1. Hemagglutinating activity of the several lectin fractions prepared from *A. heterophyllus*. The fractions designations CE, AF, GF, GQII, AIMI and AIMII are defined in Materials and Methods section.
*One Hemagglutinating Unit (UH) is the quantity of protein capable of agglutinating of 50% of a 2% suspension of erythrocytes.

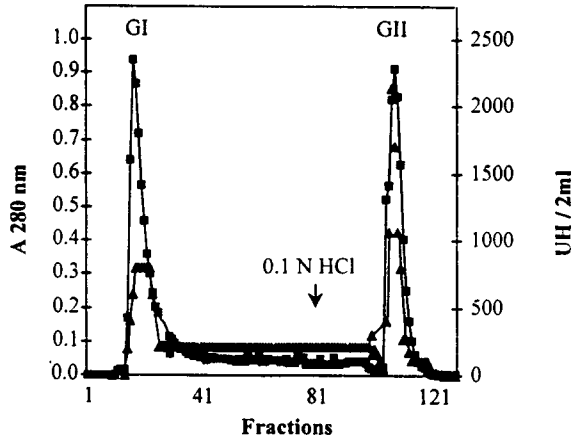

Figure 2. Guar gum affinity chromatography of the globulin fraction (GF) of A. heterophyllus (30 mg) was prepared as it is indicated in Figure 1. It was equilibrated with 0.2 M glycine, pH 8.0. The desorption step and volume of the collected fractions were as in Figure 1.
■: Absorbance at 280 nm; ▲:Hemagglutinating titer.

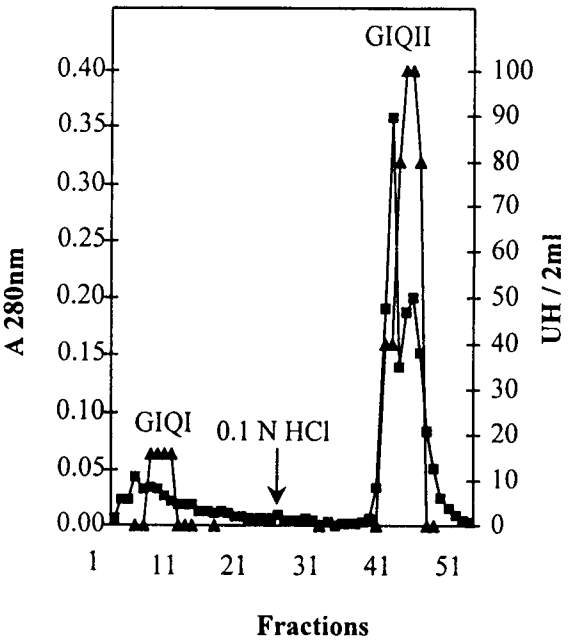

Figure 3. Chitin affinity chromatography of fraction, GI (10 mg), which did not bind to the guar gum column. The chitin column was equilibrated with PBS (0.15 M NaCl and 0.05 M sodium phosphate, pH 7.4). The desorption step was done as in Figure 1 and the fractions of 2.5 ml were collected.
■: Absorbance at 280 nm; ▲: Hemagglutinating titer.

When the carbohydrate specificity of each fraction was analyzed, GI agglutination was the only one competed by N-acetyl-D-glucosamine (Table 2). Therefore, fraction GI was chromatographically analyzed on chitin and two peaks could be discriminated: an unbound fraction (GIQI) and another one which was bound to the column (Fig. 3). The chitin bound fraction presented two peaks with different absorption values at 280 nm. Both peaks revealed identical sugar specificity and electrophoretic profile (data not shown). The one with the highest hemagglutinating activity (GIQII) was named heterophyllin (Table 2).

The AI fraction, derived from the albumins, with affinity towards mannose (Table 2) was submitted to an affinity chromatography step in an agarose-D-mannose column (Fig. 4). Two peaks resulted from this step: AIMI unbound to the column, and AIMII which was adsorbed to it. This last fraction showed a higher hemagglutinating activity than that of AIMI (Fig. 4 and Table 1). Further analysis suggested that the albumin AII, recognized as the lectin jacalin and the globulin GII are very similar to each other since properties such as the carbohydrate specificities towards galactose (Table 2), the electrophoretic profiles (Fig. 5, lanes 5 and 6) and the N-terminal amino acid sequences (Table 3) resulted coincident. However, these two lectins were isolated from protein fractions with distinct

PEAK	Inhibitor CONCENTRATION * (M)						
	mannose	α-methyl-mannoside	galactose	raffinose	N-acetyl-D-glucos-amine	N-acetyl-D-galactos amine	fructose
AI	0.5	0.125	0.25	0.25	-	0.063	-
AII	-	0.25	0.125	0.25	-	0.063	-
GI	0.5	0.031	0.125	0.25	0.5	-	-
GII	-	0.125	0.125	0.25	-	0.063	-
AIMII	0.125	0.063	-	-	-	-	
GIQII	0.5	0.125	0.25	-	0.5	-	-

Table 2. Inhibition of hemagglutinating activity of lectin fractions prepared from *A. heterophyllus* seeds by selected sugars. * The numbers indicate the minimal concentration (M) still capable of inhibiting 50% of the hemagglutinating activity. (-) indicates no inhibition.

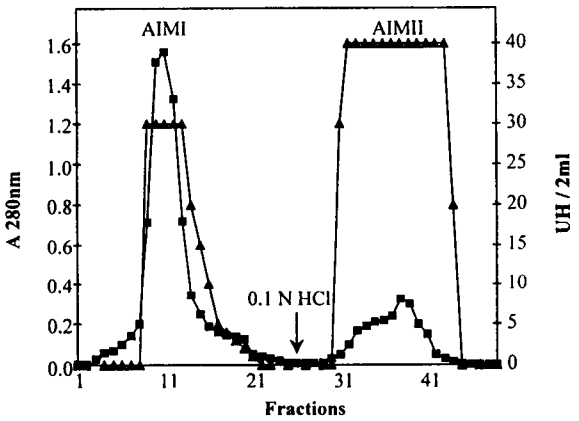

Figure 4. Agarose-D-mannose affinity chromatography of fraction AI (4 mg), which did not bind to the guar gum column. The agarose-D-mannose column was equilibrated with PBS (0.15 M NaCl and 0.0025 M sodium phosphate, pH 7.0). The desorption step was done as in Figure 1 and the fractions of 1.5 ml were collected.
■: Absorbance at 280 nm; ▲: Hemagglutinating titer.

water solubility. These could be explained by the existence of slightly modified forms of jacalin as has already been reported [5].

Partial N-terminal amino acid sequence analysis of the GII and AII fractions, judged to be jacalin isoforms, showed that only one type of polypeptide chain was detected in both fractions, corresponding to the α-subunit already described by other authors [5, 26, 27]. A second subunit (β) which contributes to the structure of jacalin [5, 26, 27] could not be detected through our analysis. This might be due to the fact that the β-subunit is not resolved on SDS-PAGE.

The lectin which binds to agarose-D-mannose (AIMII) was comparable to artocarpin, the lectin previously reported [3, 4] in seeds of *Artocarpus heterophyllus,* in that both exhibited the same carbohydrate specificity towards D-mannose. This artocarpin, indeed, has nothing in common with the artocarpin firstly described by Chaterjee *et al.* [28] which is present in the seeds of *Artocarpus lakoocha* with a carbohydrate specificity towards lactose. The N-terminal amino acid sequences of the subunits of artocarpin (AIMII, Table 3) and of the two large subunits of GIQII-designated heterophyllin could not be determined possibly due to blocking of their N-terminal amino acids.

The lectin which was isolated from the water insoluble fraction (GF), by affinity chromatography on chitin and showed sugar specificity towards N-acetyl-glucosamine is formed by three subunits of relative molecular masses (Mr) of 31.4, 18.7 and 16.3 (Fig.5). The amino terminal sequence of the first eleven amino acids from its smaller subunit (M_r 16.3) did not show any difference as compared to the amino terminal sequence of the (-subunit of jacalin (Table 3) [5, 26, 27]. It is the possibility that differences could arise if longer sequences are analyzed. When

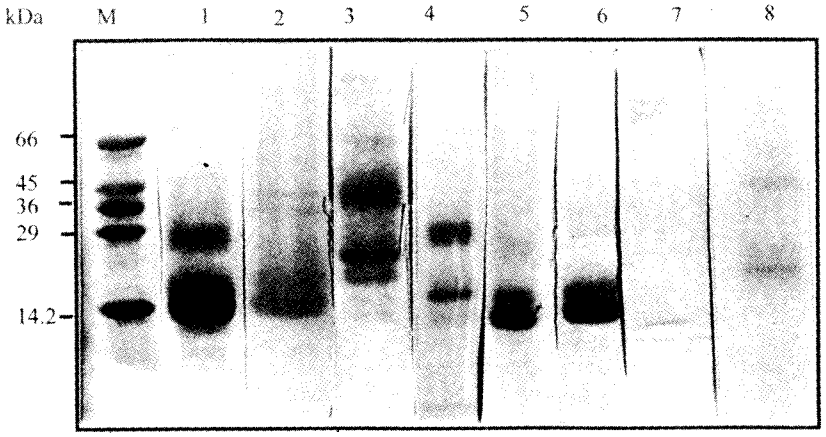

Figure 5. SDS polyacrylamide gel electrophoresis of: (1) the albumin fraction (AF); (2) globulin fraction (GF); (3) albumin fraction not bound to the guar gum column (AFI); (4) globulin fraction not bound to the guar gum column (GFI); (5) albumin fraction bound to the guar gum column (AII); (6) globulin fraction bound to the guar gum column (GII); (7) AI fraction bound to the agarose-D-mannose column (AIMII-artocarpin); (8) GI fraction bound to the column of chitin (GIQII-heterophyllin); (M) molecular mass markers (kDa). Each lane contained 20 μg of protein, except for lanes 7 and 8 that contained between 2 and 5 μg of protein.

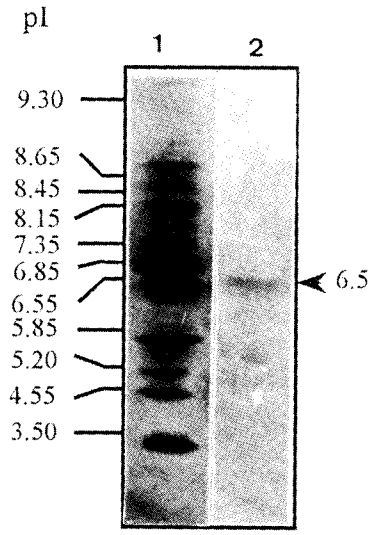

Figure 6. Analytical isoelectric focusing of the heterophyllin (GIQII) lectin in native conditions. The isoelectric points were determined by utilizing a pH 3.5-10 ampholine gradient.
(1) isoelectric point markers; (2) heterophyllin.

this lectin was analyzed by isoelectric focusing a unique band was displayed (pI of 6.5). The finding confirms the homogeneity and purity of this lectin (Fig. 5, lane 2). The new lectin that resulted different from jacalin and artocarpin was designated *heterophyllin*.

The results showing different lectin activities in seed extracts of *A. heterophyllus* indicate that care has be taken when using crude lectin preparations from seeds as a tool for different experimental purposes in order to avoid conflicting results.

Results presented in this works show new lectin activities in seed extract from *A. heterophyllus*. Different isoforms of jacalin (AII and GII) have been shown in this work. The results concerning to the lectin, we found similar to artocarpin, are still in progress to clarify such β-like subunit present in our preparations. Finally, we showed evidences on the presence of a previously undescribed lectin-designated heterophyllin-which has 3 subunit, a pI of approximately 6.5 and carbohydrate specificity to N-acetyl-D-glucosamine.

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Fraction	Molecular mass (kDa)	N-terminal sequence
GII	18.0	X K A F D D G A F T G I R E I N L S
AII	14.8	X K A F D D G A F T G I R E I N L
	18.0	X K A F D D G A F T X I ? E I N L S
	14.8	X K A F D D X A
AIMII	64.6	n.d.
	58.7	n.d.
GIQII	31.4	n.d.
	18.7	n.d.
	16.3	X K A F D D G A F T G

Table 3. Molecular mass and N-terminal amino acid sequence analysis of selected A and G lectins fractions. n.d.-non determined.
N-terminal amino acid sequence analysis of the following lectin fractions: GII (jacalin isolated from globulin fraction); AII (jacalin isolated from albumin fraction); AIMII (artocarpin) and GIQII (heterophyllin).

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