Purification of acid sphingomyelinase from human placenta: Characterization and N-terminal sequence

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Abstract Human placental acid sphingomyelinase (ASM) was purified by sequential chromatography on Con A-Sepharose, octyl-Sepharose and Matrex gel red A. Final purification to apparent homogeneity was achieved by immunoaffinity chromatography employing polyclonal anti-ASM antibodies. The antibodies also allowed specific detection of ASM by Western blotting at various stages of purification. The ASM activity was enriched about 110 000-fold over that of the crude extract, yielding an enzyme preparation with a specific activity of about 1 mmol/h per mg protein in a detergent-containing assay system. Analysis of the final preparation by SDS-PAGE resulted in a single protein band with a molecular mass of ~75 kDa, which was reduced to ~60 kDa after complete deglycosylation. Microsequencing of the purified ASM revealed the N-terminal amino acid sequence of the mature placental enzyme.

Key words: Mature acid sphingomyelinase; Human placenta; Polyclonal antiserum; Immunoaffinity purification; N-terminal sequence

1 Introduction

Acid sphingomyelinase (ASM) is a lysosomal enzyme catalyzing the hydrolysis of sphingomyelin to ceramide and phosphorylcholine. An inherited ASM deficiency results in type A and B Niemann-Pick disease (NPD), an autosomal-recessive lipid storage disorder accompanied by massive accumulation of sphingomyelin in lysosomes [1,2].

Different purification procedures from human placenta, brain and urine led to final ASM preparations of different molecular masses (30–90 kDa) with specific activities in the range of 60–800 μmol h^{-1} mg^{-1} [3–6]. In 1987 we succeeded in isolating ASM from human urine [7]. The enzyme was shown to be a monomeric ~72 kDa glycoprotein with a specific activity of ~2.5 mmol h^{-1} mg^{-1} in a detergent-containing assay system. The purified ASM also hydrolysed phosphatidylcholine and phosphatidylglycerol in a phospholipase C-like manner [7], consistent with previous findings [8–11]. Several alternatively spliced human ASM cDNAs were isolated and the genomic sequence encoding ASM was characterized [12–14]. Recently, ASM-deficient mice have been generated, thus, providing an animal model for NPD [15,16].

Special interest has focused on the role of ceramide and its metabolites in signal transduction, apoptosis, cell differentiation and proliferation. Recent studies have shown that besides a neutral membrane-associated sphingomyelinase, a lysosomal ASM plays an important role in TNF-α- and Fas-induced activation of the sphingomyelin cycle [17,18].

We recently described the production of polyclonal rabbit and goat-anti-ASM antisera raised against recombinant ASM expressed in *E. coli* [19,20]. The anti-ASM antibodies were used successfully for studies on the biosynthesis and processing of ASM in human fibroblasts and transfected COS cells, revealing distinct post-translational processing during transport to the lysosomes [19,20]. ASM is synthesized as a 75 kDa propolypeptide which is converted to a 72 kDa precursor in the ER/Golgi complex. The 72 kDa precursor form is processed to the mature lysosomal 70 kDa form in the endosomal/lysosomal compartments. However, a small and variable amount of the ASM precursor is already processed in the ER/Golgi compartments, yielding an enzymatically active 57 kDa form [20].

In order to gain more insight into the processing events of human ASM, we intended to establish an efficient procedure for the purification of ASM from readily available human placenta. As a result of the high enrichment achieved by the presented immunoaffinity purification, this procedure might be suitable for ASM purification from many other normal and pathological tissues (enzymatically less or non-active Niemann-Pick tissue). Isolation of pure N-terminally unblocked enzyme allowed, for the first time, sequencing of the N-terminal amino acids of human mature ASM, which originates from an N-terminally extended precursor protein by proteolytic processing.

2. Materials and methods

2.1. Materials

Con A- and octyl-Sepharose CL-4B were purchased from Sigma, Affi-Gel Hz from Bio-Rad and Matrex gel red A and Centricon 30 concentrators from Amicon. Peptide-*N*-glycanase F and Endo H were obtained from Boehringer Mannheim. Molecular weight standards were supplied by Amersham. PVDF membrane was provided by Macherey-Nagel and secondary antibody-alkaline phosphatase conjugates by Dianova. All other reagents were of analytical purity.

The following chromatographic steps were carried out at 4°C using a flow rate of 30 ml/h unless stated otherwise. All buffers contained 0.02% (w/v) NaN_3 as preservative.

2.2. Antibody purification and preparation of the immunoaffinity matrix

Anti-ASM immunoglobulins (IgG) were purified from polyclonal

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Abbreviations: ASM, acid sphingomyelinase; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; Con A, concanavalin A; COS, COS-1; CV1, origin simian virus 40; Endo H, endo-β-*N*-acetylglucosaminidase H; NBT, nitroblue tetrazolium; NP-40, Nonidet P-40; OG, β-D-octylglucopyranoside; PBS, phosphate-buffered saline; PVDF, polyvinylidene difluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

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goat-anti-ASM antiserum by combined $(NH_4)_2SO_4$ precipitation and anion exchange chromatography as described [21]. The *N*-glycan of the purified IgG (10–15 mg/ml) was oxidized in the presence of 60 mM $NaIO_4$ in 0.1 M sodium acetate buffer, pH 5.5 (buffer A) for 45 min at room temperature. Excess $NaIO_4$ was removed by passing the IgG solution over a desalting column (10DG, Bio-Rad). For antibody immobilization, oxidized IgGs were added to the Affi-Gel hydrazide beads (8–10 mg IgG/ml gel) previously equilibrated with buffer A and the IgG/gel slurry rotated for 8 h at room temperature. Uncoupled IgGs were removed with 10 bed volumes of buffer A, followed by 20 bed volumes of 1 M NaCl in PBS (buffer B). In order to enable elution under acid conditions (pH < 3), the hydrazone groups were reduced selectively with 15 mM $NaBH_3CN$ in 20 mM sodium phosphate buffer, pH 6.5 for 2.5 h at room temperature. After washing with the incubation buffer the immunoaffinity column (1×2 cm) was rinsed with 5 bed volumes of elution reagent (100 mM acetic acid containing 0.15% (w/v) OG) at a flow rate of 25 ml/h followed by buffer B at room temperature. The gel was stored at 4°C until use.

2.3. ASM and protein assays

Phospholipase C activity was measured with sphingomyelin and phosphatidylcholine as substrates, both ^{3}H-labeled in the choline moiety as previously described [7]. Protein was quantified by the BCA method [22] using bovine serum albumin as standard.

2.4. Digestion with glycosidases

After heating for 5 min at 95°C in 0.5% SDS, the purified enzyme (~20 ng) was digested with Peptide-*N*-glycanase F (30 mU) and Endo H (2 mU) for 20 h at 37°C in 25 mM sodium phosphate buffer, pH 7.2 containing 50 mM EDTA and 50 mM sodium acetate buffer, pH 5, respectively, in the presence of a 5-fold excess of OG over SDS.

2.5. SDS-PAGE and Western blotting

Denaturing SDS-PAGE was performed on 10% polyacrylamide gels according to Schägger and Von Jagow [23]. After SDS-PAGE, the proteins were electrophoretically transferred onto PVDF membrane using 50 mM borate buffer, pH 9, containing 15% (v/v) MeOH. The membrane was blocked for 1 h at 45°C with 2% (w/v) polyvinylpyrrolidone K90 in PBS. ASM was detected with polyclonal rabbit or goat-anti-ASM antiserum (dilution 1:1000) using anti-rabbit or anti-goat IgG alkaline phosphatase conjugate, with NBT and BCIP as substrates.

2.6. ASM purification from human placenta

Human placentae (stored for 1–2 days at 4°C) were collected from a local hospital and immediately after delivery frozen at −70°C for 2–6 months until use.

2.6.1. Homogenization and $(NH_4)_2SO_4$ precipitation. Human placentae (1.5 kg), freed of cord, membranes and adipose tissue, were sliced into small pieces and homogenized for 1 min in a Waring blender in 1.5 l of ice-cold 30 mM Tris-HCl, pH 7.2 (buffer C). Complete extraction was achieved by treating the mixture with an Ultra Turrax in the presence of 0.25% (w/v) NP-40. After centrifugation (1 h, 17 700×*g*), the supernatant (crude extract) was subjected to 30–60% $(NH_4)_2SO_4$ fractionation. The precipitate was dissolved in 600 ml of buffer C, containing 0.5 M NaCl, 0.1% (w/v) NP-40, 1 mM Ca/Mg/$MnCl_2$ and 1 μM leupeptin (buffer D).

2.6.2. Con A-Sepharose chromatography. After centrifugation (30 min, 235 000×*g*) and filtration (0.2 μm), the solubilized precipitate was applied to a Con A-Sepharose column (2.6×30 cm) equilibrated with buffer D. Unbound proteins were removed with 10 bed volumes of buffer D and bound glycoproteins eluted in the reverse direction at room temperature with a linear gradient of 0–20% (w/v) α-D-methyl-glucopyranoside in buffer D.

2.6.3. Octyl-Sepharose chromatography. Pooled fractions of the Con A-Sepharose eluate with the highest specific ASM activity were loaded onto an octyl-Sepharose column (2.6×30 cm) equilibrated with buffer C. After washing with 0.01% NP-40 in buffer C, bound proteins were eluted in the reverse direction using a linear gradient of 0.01–1.5% (w/v) NP-40 in buffer C.

2.6.4. Matrex gel red A chromatography. Pooled fractions of the octyl-Sepharose eluate containing the highest specific ASM activity were loaded onto a Matrex gel red A column (1.6×12 cm) equilibrated with buffer C containing 0.1% (w/v) NP-40 (buffer E). After washing, bound proteins were eluted in the reverse direction with a linear gradient of 0–1.5 M NaCl in buffer E.

2.6.5. Immunoaffinity chromatography. Pooled fractions of the Matrex gel red A eluate with the highest specific ASM activity were dialyzed against buffer E and applied to the immunoaffinity column (1×2 cm) equilibrated with buffer E (1 ml eluate/100 μl gel; flow rate, 2 ml/h). For binding ~20 μg enzyme, 1 ml gel (~6 mg IgG/ml gel) was sufficient. Non-specifically bound proteins were removed by sequential washing with 10 bed volumes of buffer E, 20 bed volumes of buffer B containing 0.1% (w/v) NP-40 and 5 bed volumes of buffer A containing 0.15% (w/v) OG. ASM was eluted under batch conditions by incubating the immunoaffinity gel twice with elution reagent (cf. Section 2.2) for 2 min each at room temperature (100 μl gel/2×400 μl elution reagent). The eluate was removed and adjusted to pH 5 by adding 3 M sodium acetate buffer, pH 7.5. It was concentrated using Centricon 30 (Amicon) and the buffer was exchanged with 0.15% (w/v) OG in 10 mM Tris-HCl, pH 7.2. The concentrated enzyme (~25 μg/ml) was stored in aliquots at −20°C.

2.7. N-terminal amino acid sequencing

Following SDS-PAGE, the purified ASM was electroblotted onto PVDF membrane and stained with Coomassie blue R250 according to Matsudaira [24]. The stained protein band was excised and its N-terminal sequence determined using a Beckman Sequencer (Porton 3600) with an on-line phenylthiohydantoin analyser (System Gold, Beckman).

3. Results

3.1. Purification of ASM from human placenta

Table 1 summarizes the results of a typical purification of ASM from human placenta. Sequential chromatography on Con A-Sepharose, octyl-Sepharose and Matrex gel red A resulted in a ~2400-fold enrichment of activity over the crude extract with a recovery of ~15%. An apparently pure ASM was obtained after additional immunoaffinity purification employing polyclonal anti-ASM antibodies raised against recombinant ASM. The final enzyme preparation was enriched ~110 000-fold and had a specific activity of ~1 mmol h^{-1}

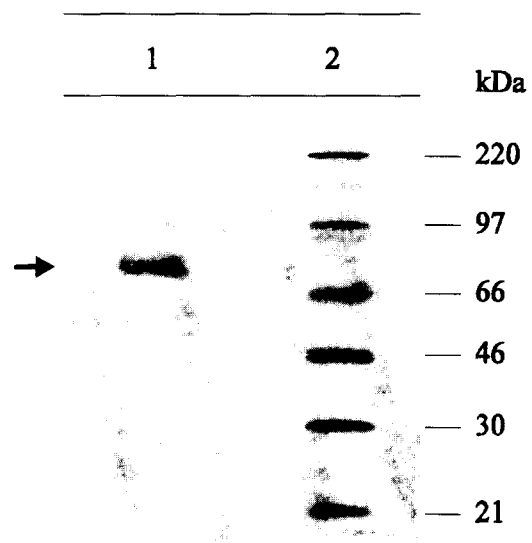

Fig. 1. SDS-PAGE of purified ASM from human placenta. Purified ASM was subjected to SDS-PAGE (10% polyacrylamide) under non-reducing conditions. Protein bands were silver stained. The arrow marks the position of the mature lysosomal ASM. Lanes: (1) purified ASM (0.25 μg); (2) molecular mass standards (0.2 μg/band), myosin (220 kDa), phosphorylase *b* (97 kDa), bovine serum albumin (66 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa) and trypsin inhibitor (21 kDa).

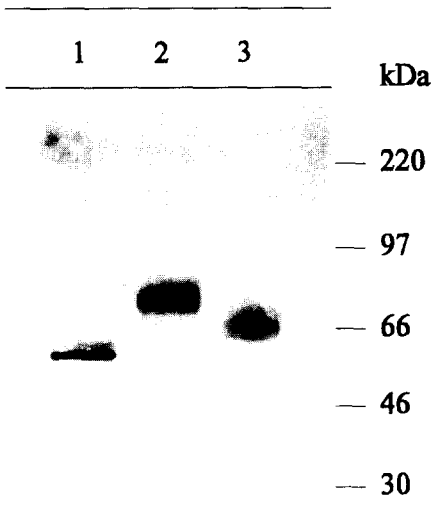

Fig. 2. Western analysis of purified ASM. Purified ASM was treated with peptide-*N*-glycanase F or Endo H. Following non-reducing 10% SDS-PAGE, the enzyme was transferred onto PVDF membrane. ASM was detected using polyclonal rabbit-anti-ASM antiserum (dilution 1:2000). Lanes: (1) purified ASM, peptide-*N*-glycanase F-treated; (2) purified ASM, (3) purified ASM, Endo H-treated (ASM/lane: ~20 ng, each).

mg^{-1}. Both values are higher than those described in previous reports on the placental enzyme [5,25].

As a consequence of the high enrichment achieved by the inclusion of immunoaffinity chromatography, this efficient step in combination with the conventional chromatographic steps first resulted in the isolation of pure placental ASM. However, the immunoaffinity step was accompanied by low enzyme recovery, presumably due to reduced affinity of the anti-ASM IgGs covalently linked to the gel matrix. The preparation of the immunoaffinity gel involved oxidation of the IgG *N*-glycan with $NaIO_4$ to enable IgG coupling to the Affi-Gel hydrazide beads and reduction of the resulting hydrazone bonds with $NaCNBH_3$, necessary for elution under acid conditions. In addition, the harsh washing and elution conditions may have contributed to loss of enzyme activity. Even in the optimized elution reagent used (100 mM acetic acid containing 0.15% (w/v) OG) the enzyme was quite labile. After only 5 and 10 min of incubation in the elution reagent, ASM activity was decreased by ~30 and ~50%, respectively. The antigen was therefore eluted batchwise and the elution time minimized to 2×2 min, allowing maximal release of bound ASM with the highest possible recovery of activity.

3.2. Properties and N-terminal sequence

SDS-PAGE of the purified placental ASM (~0.25 μg) under reducing (not shown), as well as under non-reducing conditions, followed by silver staining, revealed a single protein band with an apparent molecular mass of 75 kDa (Fig. 1). No significant contamination was detected by this method, indicating that this preparation was more than 95% pure. Microsequencing of the isolated enzyme (~4 μg) revealed the N-terminal amino acid sequence of mature lysosomal ASM (H_2N-Gly^{83}-Trp-Gly-Asn-Leu-Thr-Cys-Pro-...), which aligns with residues 83–90 of the amino acid sequence of the ASM precursor derived from the ASM cDNA.

For oligosaccharide side chain analysis, the pure enzyme was either treated with peptide-*N*-glycanase F or with Endo H and subjected to SDS-PAGE and Western blotting (Fig. 2). Completely deglycosylated enzyme was immunodetected at ~60 kDa and Endo H-treated ASM at 64–66 kDa in accordance with the results in previous reports on urinary and fibroblast ASM [7,19].

Western analysis of ASM (~20 ng) at different stages of purification using rabbit or goat-anti-ASM antiserum (1:1000) showed one significant band at ~75 kDa (Figs. 2 and 3). In addition, a faint band at ~57 kDa was detected by Western analysis of more than 30 ng of the purified enzyme (not shown) or the corresponding amount of Matrex gel red A eluate with either rabbit (not shown) or goat-anti-ASM antiserum (Fig. 3), suggesting co-purification of the placental homologue to the 57 kDa fibroblast form of ASM. Another band at ~48 kDa probably represented the deglycosylated product (not shown). The 57 kDa protein was not visible in Figs. 1 and 2, but as a faint band in Fig. 3, suggesting an amount < 5% of the total placental ASM. Further characterization of this protein was hampered by its low abundance.

The purified enzyme showed maximal activity at pH 4.6–4.7, with half maximal activity at pH 3.7–3.8 and pH 5.9–6. No ASM activity was observed below pH 3.1 and above pH 7.5 (not shown). The kinetic properties of the purified ASM were assessed using sphingomyelin as the substrate in a detergent-containing assay system (Fig. 4). The enzymatic hydrolysis of sphingomyelin followed Michaelis-Menten kinetics. The K_m and the V_{max} value were calculated to be ~25 μM and ~1.3 mmol/h per mg protein, respectively. Isoelectric focusing of the purified ASM resulted in a broad major peak of activity at p*I* 6.5 and a minor one at p*I* 7.5 (not shown).

4. Discussion

The purification of human placental ASM, as reported here,

Table 1
Purification of ASM from human placenta (1.5 kg)

Purification step	Total protein (mg)	Total activity (μmol/h)	Specific activity (μmol h^{-1} mg^{-1})	Yield (%)	Enrichment (-fold)
Crude extract	102560	923	0.009	100	1
30–60% $(NH_4)_2SO_4$ fractionation	34210	582	0.017	63	2
Con A-Sepharose	586	421.8	0.72	46	80
Octyl-Sepharose	89	277.6	3.12	30	347
Matrex gel red A	6.57	143.1	21.78	15.5	2420
Immunoaffinity chromatography[a]	0.008[b]	8.1[b]	1012.5	0.9	112500

[a]Only 50% of the total activity of the Matrex gel red A eluate was subjected to immunoaffinity chromatography.
[b]The total enzyme activity and total amount of protein of the concentrated eluate are shown.

yields an apparently homogeneous enzyme preparation as judged by SDS-PAGE followed by silver staining (Fig. 1) with a specific activity of ~1 mmol h^{-1} mg^{-1}. The ~110000-fold increase in specific ASM activity achieved by our protocol is higher than the purification factors described in previous procedures starting from human placenta, indicating inhomogeneous ASM preparations (~33000-fold, spec. act. ~0.38 mmol h^{-1} mg^{-1} [5]; ~20000-fold, spec. act. ~0.1–0.15 mmol h^{-1} mg^{-1} [26]).

Previous reports on ASM purification from other sources also resulted in significantly lower purification factors, whereas the specific activity was close to that of our preparation (urinary ASM: ~23000-fold, spec. act. ~2.5 mmol h^{-1} mg^{-1} [7]; brain ASM: ~21000-fold, spec. act. ~0.8 mmol h^{-1} mg^{-1} [4]). The differences in the enrichment factors might be due to the greater specific ASM activity described in human urine and in human brain extract.

In this report we present, for the first time, the N-terminal amino acid sequence (H_2N-Gly[83]-Trp-Gly-Asn-Leu-Thr-Cys-Pro-...) of human mature lysosomal ASM. This reveals that final proteolytic processing of the ASM precursor to the mature lysosomal enzyme occurs between the amino acid residues Phe[82] and Gly[83], three residues in the N-terminal direction from the first potential *N*-glycosylation site. This indicates that all six potential glycosylation sites are present on the mature placental enzyme. Removal of the signal peptide (presumably residues 1–46 [27]) and an N-terminal segment of 36 additional amino acids seems to be the major proteolytic processing events occurring on the monomeric ASM precursor.

The maximal possible size of the protein core of mature ASM, assuming no C-terminal proteolytic processing, is calculated to be 61077 Da using the *ExPASy* Molecular Biology Server. This theoretical molecular mass agrees well with the experimental data of ~60 kDa. Complete deglycosylation of the enzyme led to molecular mass reduction by ~15 kDa, indicating that most of the six potential *N*-glycosylation sites are utilized in vivo. The molecular mass of placental ASM appeared to be slightly higher than that for the urinary and the fibroblast enzyme [7,19]. After complete deglycosylation all enzymes showed the same molecular mass of ~60 kDa,

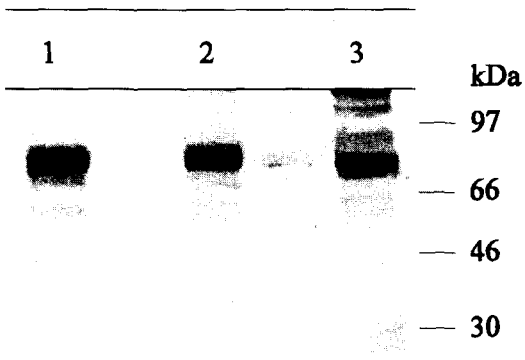

Fig. 3. Immunodetection of ASM at different stages of purification. Concanavalin A-Sepharose, octyl-Sepharose and Matrex gel red A eluate were separated by non-reducing 10% SDS-PAGE electroblotted onto PVDF membrane. ASM was detected using polyclonal goat-anti-ASM antiserum (dilution 1:1000). Lanes: (1) Matrex gel red A eluate; (2) octyl-Sepharose eluate; (3) Concanavalin A-Sepharose eluate (ASM/lane: ~30 ng, each).

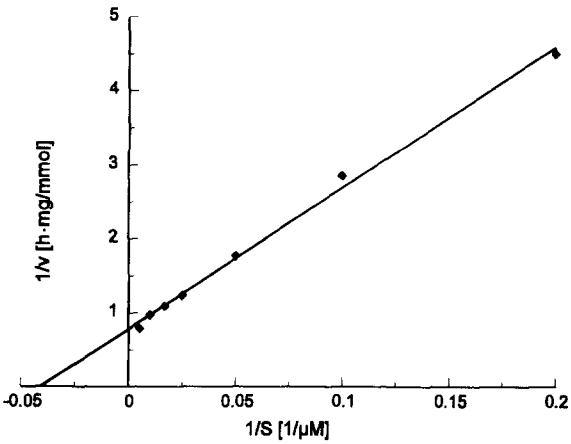

Fig. 4. Kinetic analysis of purified ASM. Purified ASM was incubated for 0.5 h at 37°C with increasing amounts of sphingomyelin in the presence of NP-40. Each assay contained: ~1.4 ng of purified ASM, [^{3}H]choline-labeled sphingomyelin (5–200 μM), 0.1% (w/v) NP-40 and 250 mM sodium acetate buffer, pH 4.8, in a total volume of 50 μl. The relationship of ASM activity values to the corresponding substrate concentrations is shown in the form of a double-reciprocal plot according to Lineweaver and Burk [26].

suggesting that these differences might be due to variable carbohydrate processing events.

Besides the major band at ~75 kDa, representing mature lysosomal ASM, a faint band at ~57 kDa was immunodetected in Matrex gel red A eluate and in the final ASM preparation (≥30 ng enzyme). This band possibly corresponds to the 57 kDa ASM form recently described in human fibroblasts and transfected COS cells [19,20]. This assumption is supported by the observation that complete deglycosylation seems to reduce its molecular mass to ~48 kDa, similar to the 57 kDa fibroblast form. However, a slow proteolytic cleavage of the mature ASM during purification, yielding the 57 kDa protein, cannot be excluded.

In summary, we have isolated apparently homogeneous placental lysosomal ASM using an efficient immunoaffinity-based purification procedure. We have characterized the pure enzyme and present for the first time the N-terminal amino acid sequence of mature mammalian ASM.

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