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Large scale protein production of the extracellular domain of the transforming growth factor-β type II receptor using the *Pichia pastoris* expression system

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Abstract

To study the (patho)physiological role of transforming growth factor-β (TGF-β), potent and selective inhibitors are necessary. Since TGF-β signaling is initiated by the high affinity binding to the type II receptor (RII), the extracellular part of RII (*sol*RII) can function as a TGF-β antagonist. *Sol*RII was cloned and large-scale protein synthesis was performed in the yeast *Pichia pastoris* expression system. Our results indicate that via this system, high levels of pure concentrated *sol*RII can be obtained. Moreover, purified *sol*RII is an active protein as shown by ELISA and bioassay. In conclusion, our large-scale protein expression procedure results in high quantities of purified *sol*RII, which is a powerful tool to study the natural role of TGF-β.

Keywords: *Pichia pastoris*; Expression system; Protein production; Transforming growth factor β

1. Introduction

TGF-β is an important regulator of cell proliferation–differentiation and matrix synthesis. Disregulation of TGF-β signaling has been implicated in several pathologies, such as the joint disease osteoarthritis (OA) [1,2]. OA is mainly characterized by cartilage loss and the formation of new bone at the joint edges, so-called osteophytes. We have previously described that exogenous added TGF-β induced OA-like side effects such as osteophytes and fibrosis [3–5]. On the other hand, exogenous addition of TGF-β into the knee joint of mice had a protective effect on the articular cartilage [6,7]. Despite extensive research on the subject, it is still unclear what the role of endogenously released TGF-β during OA is. To study the role of endogenous TGF-β during disease, potent and specific TGF-β inhibitors are necessary.

The first step in the TGF-β signaling pathway is the binding of TGF-β to its type II receptor, an interaction of high affinity [8]. The soluble, extracellular domain of RII (*sol*RII) has been shown to antagonize TGF-β [9,10]. We expressed the complete extracellular domain of the human TGF-β type II receptor in combination with a His_6-tag and the *Saccharomyces cerevisiae* α-factor secretion signal peptide in order to obtain a small (25 kDa), and

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potent soluble TGF-β antagonist. We made use of the *Pichia pastoris* protein expression system because of the advantages of eukaryotic protein processing, protein folding and post-translational modifications. Moreover, the *P. pastoris* system is an easy and cheap system with the benefit of high expression levels of recombinant protein. High-level protein production was initiated by the addition of methanol in a large-scale fermentation protocol using a 5-l tabletop bioreactor. The His_6-tagged *sol*RII was purified from the yeast supernatant using a Ni^{2+}-charged streamline-chelating column and molecular mass filters. Our results show that the obtained *sol*RII protein is a heterogeneously glycosylated protein of 25–40 kDa with a p*I* of 7. The purified *sol*RII was very potent in blocking TGF-β activity on a TGF-β responsive cell line. In conclusion, the large scale production of *sol*RII via the *P. pastoris* protein expression system will enable us to study the role of endogenous TGF-β during joint diseases such as OA.

2. Materials and methods

2.1. Construction of the yeast expression vector and transformation of Pichia pastoris

Construction of the yeast expression vector and screening of *sol*RII expressing clones are described elsewhere [11]. In short, the complete extracellular domain of human type II receptor was obtained via PCR. The 5′ primer used in the cloning procedure introduced a *Xho*I restriction site and the sequence encoding the cleavage signal of the *Saccharomyces cerevisiae* α-factor secretion signal peptide. Via the 3′ primer the sequence coding for six consecutive histidine residues (6× his-tag), a termination signal and an *Eco*RI restriction site were introduced. The PCR fragment was first cloned in the pCR-Script tm SK(+) plasmid (Stratagene, La Jolla, CA, USA), whereafter via *Xho*I–*Eco*RI digestion the *sol*RII fragment was ligated in the pPic-9 expression plasmid (Invitrogen Corporation, San Diego, CA, USA). The constructed vector (pPic-9–*sol*RII) thus contains an open reading frame coding for the *Saccharomyces cerevisiae* α-factor secretion signal peptide and the complete sequence of the extracellular domain of the TGF-β type II receptor.

P. pastoris strain GS115 was transformed using the spheroblast-method according to the manufacturers protocol (Invitrogen Corporation, San Diego, CA, USA). Briefly, the *Bgl*II-linearized pPic9–*sol*-RII construct was incubated with spheroblasts. His^+ transformants were selected by their capability to grow on media lacking L-histidine.

2.2. Production and purification of solRII

Pichia pastoris inoculum was prepared as followed. Baffled flasks containing 10% of the initial fermentation volume of Buffered Minimal Glycerol-complex medium (BMGY; 1% yeast extract, 2% peptone, 1.34% yeast nitrogen base, $4 \cdot 10^{-5}$% biotin, 100 m*M* potassium phosphate buffer, pH 6.0) were inoculated from a frozen glycerol stock. The yeast cells were grown at 30 °C, agitated at 250 rpm for 16–20 h until the OD_{600} was >5. The Bioflow 3000 benchtop fermenter (New Brunswick Scientific, NJ, USA) containing 2.5–3.5 l of fermentation basal salts medium (26.7 ml l^{-1} phosphoric acid 85%; 0.93 g l^{-1} calcium sulfate-$2H_2O$; 18.2 g l^{-1} potassium sulfate; 14.9 g l^{-1} magnesium sulfate-$7H_2O$; 4.13 g l^{-1} potassium hydroxide; 40.0 g l^{-1} glycerol) was sterilized. Temperature was set to 30 °C, agitation at 500 rpm and aeration at 0.9 vvm. The pH of the fermentation basal salts medium was adjusted at pH 5.0 with 28% ammonium hydroxide. The dissolved oxygen was maintained at 30%. The following: 4.35 ml PTM_1 trace salts (6.0 g l^{-1} cupric sulfate-$5H_2O$; 80 mg l^{-1} sodium iodide; 3.0 g l^{-1} manganese sulfate-H_2O; sodium molybdate-$2H_2O$; 20 mg l^{-1} boric acid; 500 mg l^{-1} cobalt chloride; 20.0 g l^{-1} zinc chloride; 65.0 g l^{-1} ferrous sulfate-$7H_2O$; 200 mg l^{-1} biotin; 5.0 ml l^{-1} sulfuric acid) per liter fermentation basal salts medium were aseptically added to the fermenter. The fermenter was inoculated with the overnight culture from the baffled flasks. After complete consumption of the glycerol (approximately 24 h), indicated by an increase of the DO to 100%, a 50% w/v glycerol feed containing 12 ml PTM_1 trace salts–liter was started at a rate of 18.15 ml l^{-1} initial fermentation volume. The glycerol feed was continued for 16 h.

Then, a 100% methanol feed containing 12 ml PTM_1 trace salts–liter was started at a rate of 3.6 ml l^{-1} initial fermentation volume. This feeding rate was gradually increased to a final rate of 10.9 ml l^{-1} initial fermentation volume, which was maintained for approximately 100 h.

After completing the fermentation process, the yeast cells were separated from the medium by centrifugation for 20 min at 5000 *g*. The pH of the supernatant was adjusted to 7.8 with sodium hydroxide and sodium chloride was added to an end concentration of 0.5 *M*. The supernatant was further purified by filtration over a 0.2-μm membrane filter (Schleicher and Schull, Dassel, Germany).

The recombinant *sol*RII was purified from the supernatant by means of a 6× his-tag, which has a very high affinity for nickel. To reduce non-specific binding to the column, 7.5 m*M* imidazole was added to the supernatant. The supernatant was applied to a 150 ml Ni^{2+}-charged Streamline Chelating column (Amersham Biosciences, Roosendaal, The Netherlands) in a continuous fashion for 16 h at a flow-rate of 3 ml min^{-1}. Subsequently, the column was washed with 50 m*M* sodium phosphate, 300 m*M* sodium chloride, 10% glycerol and 10 m*M* imidazole. Bound protein was eluted from the column with 300 m*M* imidazole in washbuffer and collected in 15 ml fractions. Imidazole was removed from the protein solution via a HiPrep 26/10 desalting column (Amersham Biosciences, Roosendaal, The Netherlands) using a 50-m*M* sodium phosphate and 150 m*M* NaCl buffer. The protein was further purified and concentrated using MW cut-off 100 000 and MW cut-off 10 000 Centriplus centrifugal filters (Amicon, Millipore, Bedford, USA) to a final concentration of ~60 mg ml^{-1}. The endotoxin level of purified protein was analyzed via the endochrome endotoxin detection kit (Charles River Endosafe Ltd., Kent, UK). Yeast supernatant contained an unknown interfering substance that confounded test results (results not shown).

2.3. SDS–PAGE and Western blot procedures

SDS–PAGE analysis was performed on a 12% polyacrylamide gel under denaturating conditions. Proteins were stained using a standard Silverstaining protocol. A 10-kDA protein ladder was used as a molecular mass marker.

For Western blot analysis, a benchmark 10 kDA protein ladder was used as a molecular mass marker. The SDS–PAGE gel, the blotting membrane and the Whatman paper were all equilibrated in transfer buffer (39 m*M* Tris, 48 m*M* glycine). The *sol*RII protein was blotted on a PVDF membrane (Amersham Biosciences, Roosendaal, The Netherlands) using a semi-dry blotting system during 2 h at 70 mA. Next, the PVDF membrane was blocked for 1 h with TBS–5% milk powder. After washing three times for 10 min with TBS–Tween 0.02%, the membrane was incubated overnight at 4 °C with an antibody against the extracellular part of the TGF-β type II receptor (R&D Systems, Minneapolis, USA) (1:5000) in TBS–Tween 0.02% and 3% milk powder. Then, the membrane was washed three times for 10 min with TBS–Tween 0.02%, followed by incubation with the secondary rabbit anti-goat biotin labeled Ab (1:800) in TBS–Tween 0.02% and 3% milk powder for 2 h. Subsequently, a biotin-streptavidine detection system (ABC-Kit) was used according to the manufacturers recommendations (Vector Laboratories, Burlingame, CA, USA). Bound complexes were detected by reaction with 3′,3′ diaminobenzidine (Sigma–Aldrich, Zwijndrecht, The Netherlands) in 15 ml PBS and 30% H_2O_2.

2.4. Deglycosylation of solRII

Purified *sol*RII was dialyzed against deglycosylation buffer (50 m*M* EDTA, 1% β-mercaptoethanol, 1% *n*-octylglucoside, 0.2% SDS, 50 m*M* potassium phosphate, pH 6.0), whereafter protein was denaturated by heating to 100 °C. *N*-glycosidase F (Roche, Basel, Switserland) was added (2 U^{-1} mg^{-1} protein) and the sample was incubated overnight at 37 °C. Samples were analyzed via SDS–PAGE.

2.5. Isoelectric focussing (IEF)

Phastgels IEF 3-9 (Amersham Biosciences, Roosendaal, The Netherlands) were used for IEF analysis. Proteins were visualized by silverstaining followed by isoelectric point (p*I*) determination of the recombinant protein using IEF standards ranging

from p*I* 4.5 to 9.6 (Bio-Rad Laboratories B.V., Veenendaal, The Netherlands)

2.6. Blocking capacity of solRII

The blocking capacity of the recombinant *sol*RII was determined via a TGF-β competition ELISA using *sol*RII as a capture agent. In short, ELISA plates were coated with *sol*RII (3 μg ml^{-1}; R&D systems, Minneapolis, USA) by overnight incubation at 4 °C in a sodium carbonate buffer (pH 9.6; 0.1 *M*). Non-specific binding sites were blocked with 1% gelatin–PBS for 2 h at 37 °C. TGF-β_1 was used as a standard in a concentration of 10 pg ml^{-1}–2500 pg ml^{-1} (R&D systems, Minneapolis, USA). Samples of recombinant *sol*RII were mixed with TGF-β_1 in a ratio of 1000:1–8000:1. Standards or samples were applied and incubated for 1–3 h at 37 °C. Plates were then incubated with the primary antibody (Ab), anti-TGF-β_1-Ab (1:5000 in 0.5% gelatin PBS–Tween, (R&D systems, Minneapolis, USA)) for 2 h at 37 °C. Subsequently, the secondary biotinylated Ab, anti-chicken IgY (1:5000 in 0.5% gelatin PBS–Tween, (Jackson Imunochemicals, Brunschwig Chemie, Amsterdam, The Netherlands)) was incubated for 1 h at 37 °C followed by incubation with streptavidine-poly-peroxidase conjugate for 45 min at 37 °C. Bound complexes were detected by reaction with orthophenylenediamine (Sigma–Aldrich, Zwijndrecht, The Netherlands) and H_2O_2. Adsorbance was read at 492 nm using an ELISA plate reader (Titertek Multiscan MCC/340).

2.7. Biologic activity of solRII

Now that we demonstrated that *sol*RII can block TGF-β binding to immobilized *sol*RII, we studied if *sol*RII also inhibits biologic activity of different TGF-β isoforms. Therefore, we used a modified bioassay for IL-1 [12]. In this assay we made use of the fact that TGF-β inhibits the IL-1 dependent production of IL-2 in NOB cells. The production of IL-2 was studied via the IL-2 induced proliferation of CTLL cells, present in the same culture as the NOB cells. In short, $2.5 \cdot 10^4$ NOB cells and $4 \cdot 10^3$ CTLL cells were plated in microtiter plates in RPMI medium supplemented with 5% fetal calf serum (Invitrogen Life Technologies, Breda, The Netherlands). IL-1β was added (12.5 pg) and standards (TGF-β) (R&D systems, Minneapolis, USA) or samples (1.5 ng ml^{-1} TGF-β_1, -β_2 or -β_3 together with 30 μg ml^{-1} *sol*RII or 30 μg ml^{-1} bovine serum albumin) were added. Cells were then cultured overnight at 37 °C and 5% CO_2 before 1 μCi ml^{-1} ^{3}H-thymidine was added. Cells were cultured for another 4 h, after which the cells were harvested and the incorporated radioactivity was measured using a liquid scintillation counter. All values were normalized to controls to which no TGF-β was added.

3. Results

3.1. Protein production–purification

To study the natural role of TGF-β during disease the extracellular part of the type II receptor can be used as a TGF-β antagonist. For these studies large amounts of highly purified *sol*RII in high concentrations are necessary. Here we describe the large scale production of a secreted protein in the *Pichia pastoris* expression system. Via a one step purification protocol using a Ni^{2+}-Streamline Chelating column and one further concentration step, we were able to concentrate the *sol*RII from the initial 5 l to approximately 1 ml. Per 5 l fermentation yeast culture ~100 mg pure protein was obtained.

3.2. SDS–PAGE and Western blot procedures

We characterized the recombinant *sol*RII as produced by *Pichia pastoris*. Therefore, purified *sol*RII was analyzed via SDS–PAGE. Recombinant *sol*RII showed a pattern of protein bands ranging from about 25–40 kDa (Fig. 1a). The faint high molecular mass bands are most likely alcohol oxidase, a native *Pichia pastoris* protein, which is strongly upregulated by the methanol, added during protein production.

Western blot analysis using a specific antibody against the extracellular domain of the TGF-β type II receptor, shows a similar staining pattern of 25–40 kDa, demonstrating that the purity of the produced recombinant *sol*RII is approximately 95% (Fig. 1b).

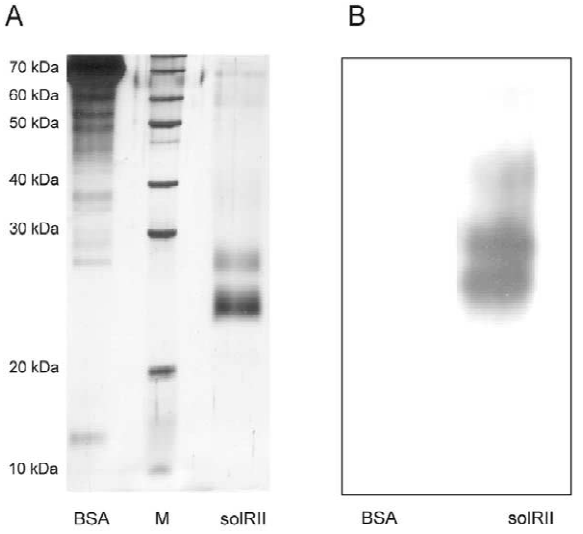

Fig. 1. SDS–PAGE and Western blot analysis of recombinant *sol*RII produced by *P. pastoris*. (A) SDS–PAGE analysis was performed on a 12% polyacrylamide gel under denaturating conditions. Proteins were visualized via a standard silverstaining. A 10 kDa protein ladder was used as a molecular mass marker. (B) Westernblotting was performed on PVDF membrane using a semi-dry blotting system. *Sol*RII was detected using an antibody against the extracellular part of the TGF-β type II receptor. Visualization of bound complexes was done by reaction with 3′,3′ diaminobenzidine and H_2O_2. A benchmark 10 kDa protein ladder was used as a molecular mass marker.

The antibody used did not cross react with a BSA preparation contaminated with other proteins.

3.3. Deglycosylation of solRII and pI

A shown by the SDS–PAGE and Western blot the recombinant *sol*RII as produced by *Pichia pastoris* did not result in a distinct band but a smear of 25–40 kDa was observed. To investigate if this smear was the result of heterogeneous glycosylation we treated *sol*RII with *N*-glycosidase F. As Fig. 2 shows, deglycosylation resulted in one distinct band of 22 kDa, indicating that *sol*RII produced by *Pichia pastoris* is a heterogeneously glycosylated protein. The observed molecular mass of 22 kDa is in line with the expected molecular mass based on amino acid sequence. IEF analysis of purified *sol*RII showed that the pI of the protein was 7.0 (results not shown).

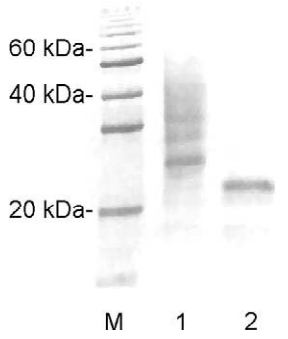

Fig. 2. SDS–PAGE analysis of recombinant *sol*RII produced by *P. pastoris* before (lane 1) and after (lane 2) deglycosylation with *N*-glycosidase F. SDS–PAGE analysis was performed on a 12% polyacrylamide gel under denaturating conditions. Proteins were visualized via a standard silverstaining. A 10 kDa protein ladder was used as a molecular mass marker.

3.4. Blocking capacity of solRII

We proceeded to determine the blocking capacity of *sol*RII with a competition ELISA. A 1000–8000-fold excess of *sol*RII was used to block 2.5 ng ml^{-1} TGF-β. To completely neutralize TGF-β, an 8000-fold excess of *sol*RII was required, as is shown in Fig. 3. *Sol*RII in excess of 4000 and 2000-fold prevented detection of TGF-β in the order of 96 and 84%, respectively. A 1000-fold excess of *sol*RII was still able to neutralize 68% of TGF-β.

3.5. Biologic activity of solRII

As can be seen in Fig. 4, *sol*RII was able to fully inhibit TGF-β1 and -β3 activity in the bioassay. In contrast, *sol*RII was unable to antagonize TGF-β2 activity. BSA had no inhibitory effect on TGF-β isoform mediated inhibition of proliferation.

4. Discussion

Disregulation of the TGF-β pathway has been described in many different pathologies such as RA and OA. In order to investigate the endogenous role of TGF-β during joint disease, potent and specific inhibitors are necessary. The soluble, extracellular domain of the TGF-β type II receptor (*sol*RII) has been shown to bind TGF-β and neutralize TGF-β

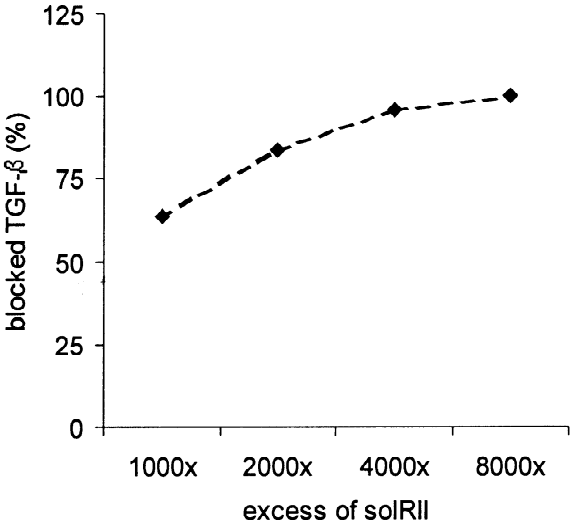

Fig. 3. Determination of *sol*RII activity. The activity of freshly produced *sol*RII was determined with a competition ELISA. ELISA plates were coated with *sol*RII. Samples containing 2.5 ng ml^{-1} TGF-β were mixed with 2.5–20 μg ml^{-1} *sol*RII and applied to the plate. The TGF-β bound to the coated *sol*RII was visualized via an anti-TGF-β Ab. The percentage of blocked TGF-β is shown.

activity [9,10]. Not only does *sol*RII bind TGF-β with high affinity but due to its small size *sol*RII is able to penetrate dense matrixes such as cartilage.

The systemic blocking of endogenous TGF-β during experimental OA will require large quantities of protein. Therefore, we opted for large scale fermentation in combination with the *Pichia pastoris* protein expression system to obtain large amounts of pure protein. Since yeast is an eukaryotic organism the expression system has all the benefits of eukaryotic post translational modifications such as protein folding and glycosylation as compared to prokaryotic systems.

Through the introduction of the His_6-tag the *sol*RII could easily be purified via a one step affinity chromatography using a Ni^{2+}-charged streamline chelating column. We opted for a wide column filled with a relative high ratio of column material to protein, in order to purify all His-tagged protein in one run. During the fermentation process, protein synthesis was induced by the addition of methanol, which also caused the strong upregulation of alcohol oxidase, a native *Pichia* protein. Despite stringent washing with imidazole the *sol*RII protein sample was still contaminated with AOX. We made use of the difference in MW between AOX (100 kDa) and *sol*RII (25 kDa) to separate the two proteins. After extreme concentration to ~60 mg ml^{-1} our results show that only very little AOX was present in the sample (<5%). From one fermentation run we were able to purify approximately 100 mg pure *sol*RII protein.

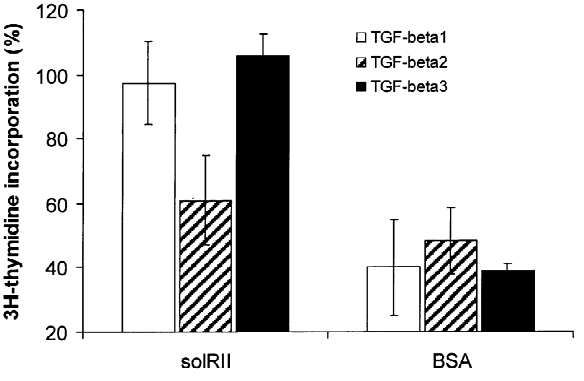

Fig. 4. Determination of *sol*RII bioactivity in neutralizing TGF-β1, -β2 and -β3. Samples containing 1.5 ng ml^{-1} TGF-β were incubated in the presence of 30 μg ml^{-1} *sol*RII or BSA as a control. The NOB–CTLL bioassay was used and the ^{3}H-thymidine incorporation which is inhibited by TGF-β was measured using a liquid scintillation counter. The ^{3}H-thymidine incorporation in the absence of TGF-β is stated as 100%.

Our results indicate that *sol*RII produced by *Pichia pastoris* is a heterogeneously glycosylated protein, which is in line with earlier reports [9,10]. The *sol*RII we produced via the *Pichia pastoris* expression system has neutralizing activity. A 4000-fold excess of recombinant *sol*RII to TGF-β was able to neutralize 96% of TGF-β activity as determined via ELISA. This is well in range with other studies in which 1600–20 000-fold excess of *sol*RII was needed to fully inhibit TGF-β activity [10,13]. The results from the bioassay show that *sol*RII is a potent inhibitor of TGF-β1 and -β3 activity. In contrast, *sol*RII was unable to antagonize TGF-β2 activity, a finding also described by others [9,10,14]. This is probably due to the high homology in protein structure between TGF-β1 and -β3 as compared to TGF-β2 [15,16]. Moreover, for TGF-β2 to bind RII and to be biologically active the presence of the type III receptor is required [14].

Here we describe the large scale production of

*sol*RII, a potent and specific TGF-β antagonist. The *P. pastoris* expression system combined with an optimal fermentation process resulted in the production of high levels of *sol*RII. The effective purification procedure enabled us to obtain *sol*RII in extremely high concentrations. *Sol*RII can now be used to study the role of endogenous TGF-β during disease.

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