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BIOCHEMICAL AND *IN SILICO* CLOTTING ACTIVITY OF LATEX FROM *ASCLEPIAS CURASSAVICA.L*

KARPAGAM .N*, VISWANATHAN.S, PRABHU.S, SULOCHANA SOMASUNDARAM AND SIVANANDHAM.M

Department of Biotechnology, Sri Venkateswara College of Engineering , Sriperumbudur-602105, Tamil Nadu, India.

ABSTRACT

The crude latex extract of *Asclepias curassavica* contains many proteins and exhibits strong proteolytic activity. The study was done to identify the clotting factor present in the latex of *Asclepias curassavica* and to analyze the thrombin like activity of the clotting factor. In this study the plant latex from *Asclepias curassavica* was collected and extracted the proteins. Proteolytic assays and inhibitor assays were carried out in the crude sample extract. The docking studies were carried out using Bioinformatics tools. The plasma clotting time was reduced from 143 seconds to 56 seconds in the presence of the crude latex extract. Reduction in coagulation time was observed with increase in the concentration of crude extract. *In-silico* studies showed that the cysteine protease of *Asclepias curassavica* interacts with the coagulation factors. The Factors VIII and XI there by leading to clot formation. Hence, from this research, it was proved that the compounds, Asclepain cI and cII, were readily involved in the clotting activity and were also found to reduce the time of coagulation.

KEYWORDS: ***Asclepias curassavica,*** **Asclepain cII, Blood coagulation, Clotting Factor VIII and Factor XI, Cysteine protease, Thrombin.**

KARPAGAM .N

Assistant professor, Department of Biotechnology,Sri Venkateswara College of Engineering, Sriperumbudur-602105, Tamil Nadu, India.

Corresponding author

INTRODUCTION

Proteases are enzymes that direct specific and selective modifications of proteins, leading to the activation of proenzymes, digestion of fibrin clots, defence against plant pathogens (especially fungi and insects), sanguineous coagulation[1-3], analgesic effects[4-7] and in the induction of apoptosis[8-10]. The proteases play a major role in the biotechnology[11-14] because they maintain their enzymatic activity within a wide range of pH and temperature [15-20]. Presently, the use of proteases in different areas of the industry varying from synthesizing modified proteins for the food industry to baking and beer elaboration, cheese production, cleaning of surgical supplies and biomedical equipments[21, 22, 23, 24]. The presence of certain enzymes like chitinases and proteases in latex vacuoles suggest that they may help plants for defence against pathogens, parasites, and herbivores by attacking the invader once the plant cell is lysed[25]. Purification and characterization of proteases present in lattices of several members of the Asclepiadaceae family, was found to be reported[26-34]. The stems of *Asclepias curassavica* exude latex when superficial incisions were made onto the stem, which has ample folk-medicinal uses as emetic, vermifuge and antigonorrheic[35, 36]. Like most milkweeds, it is perennial and has opposite leaves and milky sap[37]. Papain is a small protease with broad substrate specificity and is one of the many cysteine proteases identified long ago in the latex of various plants[9]. The main proteolytic component, named asclepain cI, a papain-like endopepdidase was isolated from the latex[38] and reports were documented to show that acceleration in clotting activity was caused by these cysteine proteases including asclepain cII, which has higher specific activity than asclepain cI[40]. Their activity was specifically inhibited by the cysteine-protease inhibitor E-64[10]. These proteases, which were isolated from several plant latexes, have been known to interfere in blood coagulation due to their thrombin like activity[7, 8]. Similar proteases like ficin, a cysteine protease isolated from *Ficus carica*, was found to shorten the prothrombin time thereby accelerating clotting activity[5]. Other plant cysteine proteases such as Ervatamin B from *Ervatamia coronaria* and a mixture of cysteine proteases isolated from *Carica papaya* showed similar activities[6, 7]. Hence, further analysis in the clotting activity of *A.curassavica* latex has been proposed and done.

MATERIALS AND METHODS

Collection of latex

A. curassavica L. ‘‘scarlet milkweed’’ (Asclepiadaceae) is an erect, evergreen perennial sub shrub with woody base. Like most milkweeds, it has opposite leaves and milky sap.The latex was collected from the tender parts of *Asclepias curassavica.* By breaking the petioles, 5 ml of the dripping latex was collected and stored at 4°C.

Protein extraction

Latex (2 ml) obtained by superficial incisions of petioles, was collected in centrifuge tubes and 1ml of the following solution were added, 2ml of 0.175M TrisHCl (pH 8.8), 2ml of 5% SDS, 2ml of 15% glycerol and mixed with 3ml of acetone. It was centrifuged at 6000 rpm for 10 mins at 4°C. The weight of the pellet was found out and the pellets were washed with buffer.

Electrophoresis (SDS PAGE)

Purified samples of asclepain cII were analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) with Tris–glycine cathodic buffer in 10%polyacrylamide gels[39, 40]. Current was kept constant at 40 mA during stacking and then increased to 60 mA and was run for 40 min. The Gel obtained was stained with coomassie brilliant blue and destained with 7% acetic acid.

Protein Estimation

The estimation of protein, in the extract, was done using Lowry’s method[54]. The phenolic group of tyrosine and tryptophan residues (amino acid) which are present in a protein produced a bluish purple colored complex,

when measured at 660 nm wavelength, with Folin- Ciocalteau reagent. Thus the intensity of color depended on the amount of these aromatic amino acids present and would thus vary for different proteins.

Proteolytic assay

Proteolytic assay of the enzymes was done using casein. The reaction mixture was prepared with 1.1 ml of 1% casein solution in 0.1 ml enzyme solution. The reaction was carried out at 42°C for 24 hours and it was stopped by the addition of 1.8 ml 5% trichloroacetic acid, then test tubes were centrifuged at 3000 rpm for 20 min and the absorbance of amino acids and soluble peptides was measured at 280 nm. The control assay was performed without enzyme in the reaction mixture and used as reference. An arbitrary enzyme unit (Ucas) was defined as the amount of enzyme that produces an increase of one absorbance unit per minute in the assay conditions. Azocasein was the substrate used in inhibition assays with 1,10-phenantroline. The crude extract, obtained without EDTA (0.15 ml) was added to 0.25 ml of 1% (w/v) azocasein in 0.1 M Tris–HCl buffer at pH 8.5 containing 12mM cysteine, at 42°C. After incubation for 5 min, the reaction was stopped by the addition of 1 ml of 10% trichloroacetic acid, then centrifuged at 5000 rpm for 15 min, the supernatant (0.9 ml) was transferred to a test tube containing 1 ml of 1 M NaOH and the absorbance was read at 440 nm[37].

Inhibitor assay

Inhibitor assay was carried out on the crude enzyme extract. The reaction mixture was prepared by mixing 0.1 ml of reagent buffer, inhibitor, protease and enzyme diluent solution with 1% casein containing 12 mM cysteine in a 0.1 M Tris–HCl buffer (pH 8.5).After incubation at 37ºC for 10 min, protease inhibitor complex and TCA were added and the absorbance of the supernatant was read at 660nm. An arbitrary enzyme unit was defined as the amount of enzyme that produces an increase of one absorbance unit per minute in the assay condition was taken.

Thrombin Time Assay

2ml blood was collected and mixed with EDTA and centrifuged for plasma separation. The plasma was taken in three ependoffs, in one of which the protein sample was added and other ependoff was added with inhibited sample. The control sample containing only the plasma was observed for the thrombin time calculation.

Fibrinogen Polymerisation Assay

100ml of nutrient blood agar plates was prepared. After solidification three wells were punched, the first well with inhibited protein sample was added, the second well with uninhibited protein sample was added and the last well with no protein sample serve as control. They were incubated for 2-3 hours and observed for the clear zone formation in the Fibrinogen Polymerisation Assay.

Concentration Dependant Coagulation Assay

Fresh Human blood was mixed with 0.11M Tri Sodium Citrate in the ratio of 9:1. The mixture was centrifuged at 1000 rpm for 10 minutes. The supernatant (platelet poor plasma) was pre-warmed to 37ºC. To 300µL of supernatant different concentrations of crude latex extract 5, 10, 15 and 20 mg/ml was added in 0.01M TrisHCl buffer and incubated for 1 minute. The time taken for coagulation and percentage reduction of time for clot formation was recorded in the Concentration Dependent Coagulation Assay.

In silico studies

In silico studies were carried out using bioinformatic tools such as CLUSTALW, BLAST, CLUSPRO and modeller software, wherein the thrombin like activity of the cysteine protease asclepain cII present in *Asclepias curassavica* was studied by comparing it with other plant cysteine proteases known for accelerating clotting activity. Docking studies were carried out between asclepain cII and the blood coagulation factors VIII and XI which are activated by thrombin in the blood coagulation cascade.

Sequence Similarity Studies

The Asclepias cII sequence (cysteine protease) was obtained from NCBI. It was compared with thrombin like enzymes found in other plant proteases which are used in traditional medicine for blood coagulation. The tool used was CLUSTALW. The sequence similarity was recorded.

Structure Generation

Asclepain cII sequence obtained from NCBI was used to obtain a sequence with highest similarity using BLAST tool. The structure of this sequence was found with its PDB id in RCSB. This structure was used as a template to generate the structure of Asclepain cII using Modeller software. The structure of Asclepain cII was used for the docking studies.

Docking Studies

The structure of Asclepain cII obtained from the Modeller software was used for docking studies. The online tool CLUSPRO was used to find the binding region of Asclepain cII with coagulation factors VIII and XI to check for the thrombin like activity of the enzyme.

RESULTS

Latex proteins were extracted using TrisHCl. The weight of the proteins extracted was found to be 0.0192g/ml of latex. SDS PAGE showed clear bands and the lanes were observed. Lane A-Tris extracted proteins; Lane B- TrisHCl extracted proteins, Lane C-protein marker (14kDa-116kDa)

Proteolytic and inhibitor assays

Absorbance at 440nm for Proteolytic assay and 660nm for Inhibitor assay was analysed. Figure 1 shows the Tyrosine standard curve for Proteolytic assay and Figure 2 shows the Tyrosine standard curve for Inhibitor assay.

Figure 1
Tyrosine standard curve for Proteolytic assay.

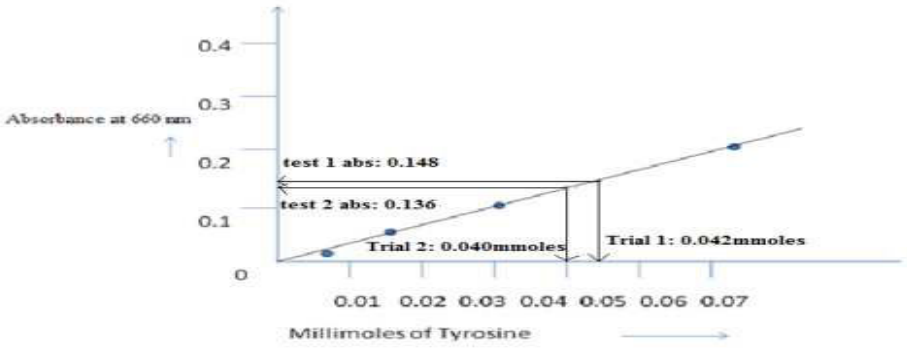
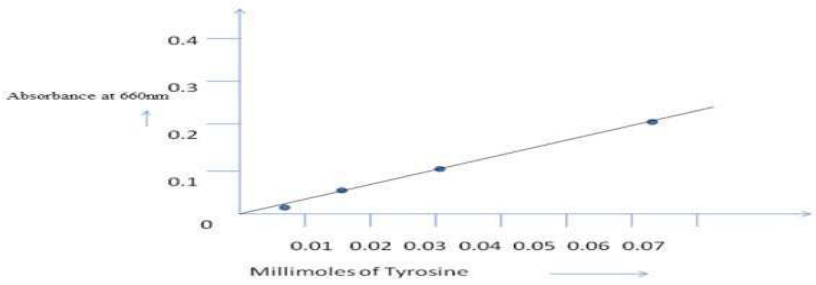


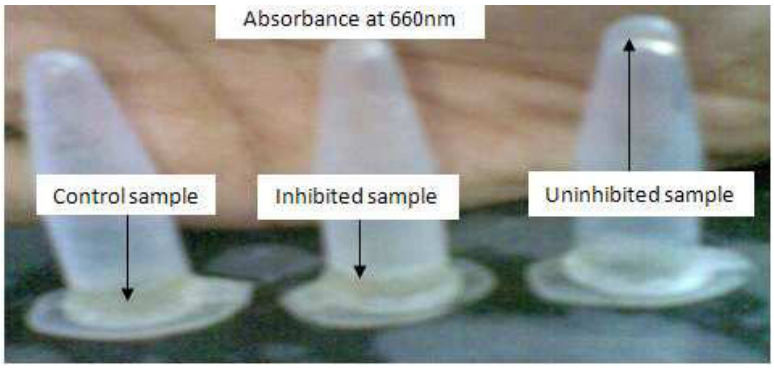
Figure 2
Tyrosine standard curve for Inhibitor assay.



From the test absorbance value of 0.198 the amount of Tyrosine released was found to be 0.042 millimoles. Units/ml enzyme = (Test absorbance value x total assay volume) / (ml of enzyme used x minutes of incubation x volume taken for absorbance)
Units/ml enzyme = (0.042 x 11) / (1 x 10 x 2) = 0.023 units/ml enzyme
Units/g solid = (units/ml enzyme) / (weight of solid / volume of assay used)
= (0.023) / (0.0303/2)
= 1.536units/g of solid
From the test absorbance value of 0.136 the amount of Tyrosine released was found to be 0.040 millimoles.
Units/ml enzyme = (Test absorbance value x total assay volume) / (ml of enzyme used x minutes of incubation x volume taken for absorbance)
Units/ml enzyme = (0.040 x 11) / (1 x 10 x 2) = 0.022 units/ml enzyme
Units/g solid = (units/ml enzyme) / (eight of solid / volume of assay used)
= (0.022) / (0.0303 /2)
= 1.452 units/g of solid

Thrombin time calculation assay

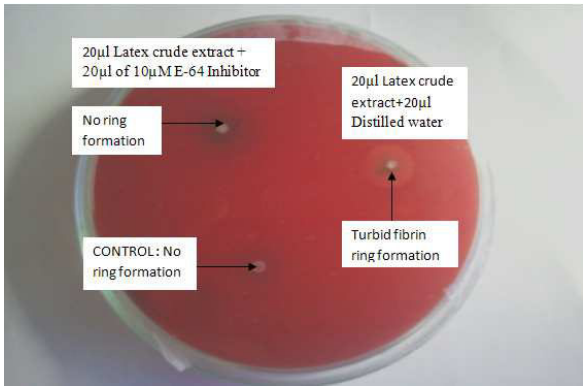
Figure 3
Thrombin time coagulation assay.



The thrombin time of the control sample containing only the plasma was 143 seconds which was reduced to 56 seconds in the uninhibited sample containing the plasma and the latex. The inhibited sample showed decrease in coagulation time as in Figure 3.

Fibrinogen polymerization assay

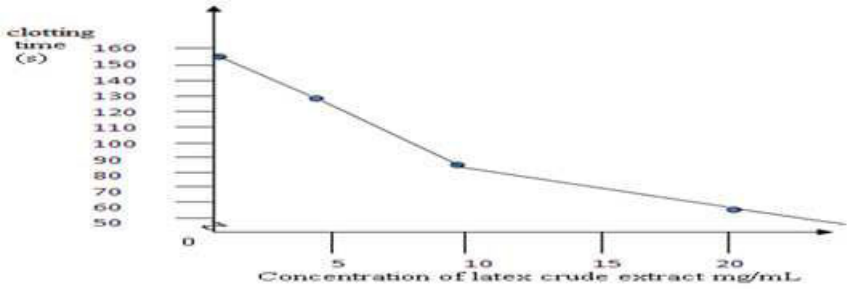
Figure 4
Blood agar plate fibrinogen polymerization assay.



Well loaded with 20 µl crude latex extract proteins+20 µl distilled water showed a turbid fibrin ring formation of 1.1mm diameter indicating the thrombin like activity of the latex proteins. There was no turbid fibrin ring formation in the wells containing 20 µl crude latex extract proteins + 20 µl of 10µM E-64 inhibitor indicating the inhibition of thrombin like activity of the latex due to the inhibitor. The control well containing distilled water showed no fibrin ring formation as in Figure 4.

Concentration dependent coagulation assay

Figure 5
Concentration dependant coagulation assay.



The concentration dependant coagulation assay, as indicated in Figure 5, showed the decrease in clotting time with increase in concentration of the crude latex extract.

Bioinformatics- Sequence similarities between Asclepias curassavica and Carica papaya

Figure 6
Bioinformatics - Sequence similarity between Asclepias curassavica and Carica papaya.

Figure 6 shows the sequence similarities between *Asclepias curassavica* and *Carica papaya*. The tool used was CLUSTALW.

Bioinformatics – Asclepias curassavica structure generation

Figure 7
BIOINFORMATICS–Asclepias curassavica structure generation.

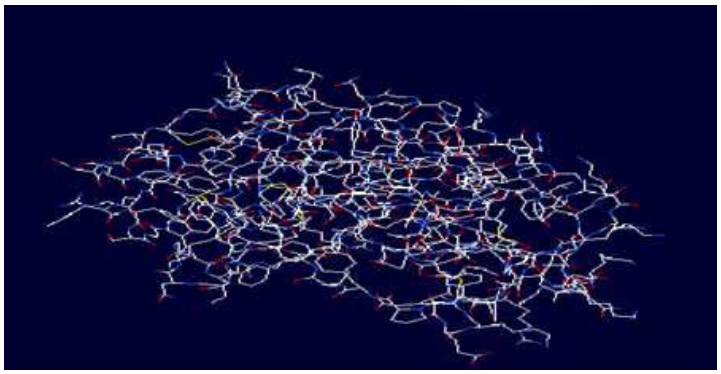


Figure 7 shows the structure generation for *Asclepias curassavica.* The tool used was Modeller.

In silico studies - Docking studies

Figure 8 shows the docking studies between *Asclepias curassavica* and factor VIII. The OE_2 atoms of the residue GLU 32 of the ligand *Asclepias curassavica* interacts with the HH_{11} and HH_{21} atoms of the ARG 541 residue of the receptor factor VIII indicating the thrombin like activity of *Asclepias curassavica.*

Figure 8
Docking studies between Asclepias curassavica and Factor VIII.

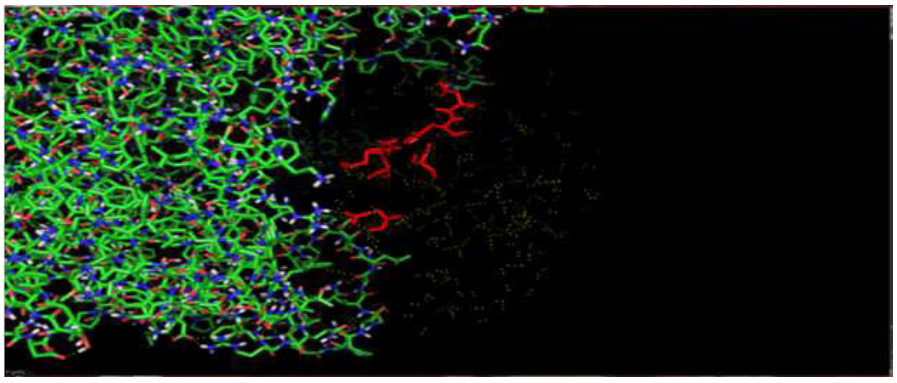


Figure 9
Docking studies between Asclepias curassavica and Factor XI.

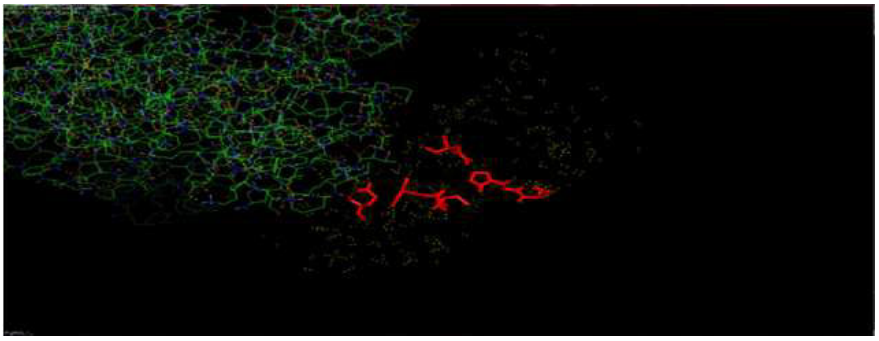


Figure 9 shows the docking studies between *Asclepias curassavica* and factor XI. The OE_2 atom of the residue GLU 32 of the ligand *Asclepias curassavica* interacts with the HH_{22} and HEatoms of the ARG 250 residue of the receptor factor XI and the OE_1 atom of the residue GLU 32 of the ligand interacts with HE atom of the residue ARG 250 of the receptor indicating the thrombin like activity of *Asclepias curassavica.*

DISCUSSION

Latex is a milky sap produced by more than 12,500 plant species spanning 20 families[46]. It is proposed that natural latex has a protective function, sealing wounds, acting as a barrier to microorganisms, and discouraging herbivory[47]. In addition to a wide range of low molecular weight polypeptides, several other proteins and enzymes have been identified in the latices of laticiferous plants[50, 51]. Latex is produced in specialized cells known as laticifers, which arise in two distinct ways depending on the species[48,52]. Nonarticulated laticifers are multinucleate cells that tend not to fuse into vessels[49]. Phenolic extraction resulted in the protein pellets which contained the majority of proteins[11]. The sequences of these cysteine proteases were found to be similar to the sequence of the cysteine protease asclepain cII of *Asclepias curassavica* which indicated that the thrombin like activity of the latex was attributed to asclepain cII [6, 7]. Factor XI gets converted to factor X_a which was involved in clotting. This factor was inhibited by antithrombin III[5]. Experiments revealed that papain like cysteine proteases served to protect the plants from insects and wounding. However, these defence proteins may also be toxic in nature[9]. The cysteine protease of *Asclepias curassavica* was placed under the papain family[8]. All isolates were evaluated for their cytotoxic activity and the strongest cytotoxic activity was against cancer cell lines[12]. Asclepain cII, the new protease isolated and purified from the latex of *Asclepias curassavica*, could be useful in pharmaceutical and biotechnology industries due to their wide ranges of activity over temperature and pH[43, 44]. Generally *Asclepias curassavica* was a traditional medicinal plant used by tribal people in the western ghats, India, to treat piles, gonorrhoea, roundworm infestation and abdominal tumours[13, 53]. The important loss of proteolytic activity at 60–70°C of asclepain cII denotes a different behavior from other proteases studied in our laboratory[27, 28, 30, 31]. Thermal behavior of these enzymes is a useful property, since it could be easily inactivated when used in the food industry, so that active enzyme is not ingested[45]. Phenol, being more acidic with a pH range of 5-6 can extract the basic proteins present in the latex with higher efficiency than Tris HCl (pH 8.8). There are presence of basic proteins in the latex of *Asclepias curassavica,* with potential benefits and harmful effects[1]. The plant *Asclepias curassavica* was known to be toxic and was also reported case of corneal edema in a sixty year old male patient who had hazy vision in the left eye due to contact with the white, milky latex of this plant and it was also seen to cause dermatitis in susceptible individuals[3]. Proteolytic activity was carried out using casein as the substrate. Inhibitor studies revealed that 1.2070 units/g of the latex proteins were inhibited in the presence of E-64 inhibitor (10mM concentration) which is specific for cysteine proteases. This indicated that the proteolytic activity of the latex is due to the presence of cysteine proteases[10, 14]. 20mg/ml of the latex proteins reduced the time taken for coagulation by 64.5% [2]. The sequence similarities of *Asclepias curassavica* cysteine protease was compared with other plant cysteine proteases exhibiting thrombin like activity such as *Ervatamia coronaria* and *Carica papaya.* The results obtained above indicates that *Asclepias curassavica* exhibits thrombin like activity[6,7].

CONCLUSION

The cysteine proteases present in the latex of *Asclepias curassavica* was found to accelerate the blood coagulation process. The documented results revealed that the cysteine

proteases present in the latex were responsible for its thrombin like activity. The commercially available procoagulants may cause carcinogenic effects when used in excessive amounts. Apart from just being a coagulant, the latex of *Asclepias curassavica* also has some anti-cancerous properties. This usage of only the latex's cysteine protease may reduce the side effects such as allergy reactions caused by the latex and hence, with further studies, related to the toxicology of the compound, the compound's minor ill effects can be reduced effectively and it can be used as a potential agent to increase the coagulation of the blood and also to effectively reduce the time of coagulation.

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