



New sources of papain: SEM and SDS-PAGE analysis to determine the natural tenderizer from papaya latex and senesced leaves

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ABSTRACT This study aims to determine the effectiveness of papaya-fruit latex and yellow-senesced leaves as a natural and organic tenderizer. The fruit and leaves of the plant were ground to powder, while 0 g, 10 g, 15 g and 20 g variations were used to cover 50 g of meat for 4 h. Subsequently, the Bradford and Kjeldahl methods were used to determine the protein content, while the protein profile was analyzed using SDS-PAGE and confirmed using a Scanning Electron Microscope (SEM). The results showed that the protein concentration in mutton after fruit latex treatment was 41%, which was higher than the concentration of beef at 29.86%. Furthermore, the beef lost protein bands and its molecular weight fell from 225 kDa to 86 kDa, while the mutton experienced a reduction from 100 kDa to 65 kDa, which was significantly smaller than for raw meat. A single protein band was also observed at 21.6 kDa in the sample, indicating the presence of papain enzyme protein. Meanwhile, the SEM results showed that collagen and myofibril in the muscles were damaged in the treated meats. Based on these results, treatment with papaya fruit latex and yellow papaya leaves increases the tenderness of meat.

KEYWORDS papain; latex papaya; senesced leaf papaya; meat tenderizer

1. Introduction

The texture of meat is characterized by its muscle type, and the level of physical activity (Ertbjerg and Puolanne 2017; della Malva et al. 2017) such as beef and mutton with a hard texture, which requires a long cooking process (Suleman et al. 2020). Meat tenderness is also determined by the myofibrillar structure or the state of muscle contraction, connective tissue content, degree of cross-binding, and the protein water-binding capacity (Istrati 2008). Furthermore, improper processing when cooking beef and mutton leads to a firmer texture and reduces the protein content as it dissolves during the boiling process (Bhat et al. 2018). The majority of people in the community also mix certain parts of plants with meat tenderizing activities, such as papaya (Welde and Worku 2018; Sukada et al. 2019), which contains papain, a protein degrading enzyme.

The papaya plant is well known for the meat tenderizing ability of the fruit and young leaves, and it tenderizes meat by breaking down the protein microstructure (Kartika et al. 2019). Meanwhile, old papaya leaves are not

used as a meat tenderizer, although they have gone through a senescence process, which increases the protease enzyme level. The old leaves also contains more protease enzyme than green leaves (Agrahari and Sharma 2014). Conversely, the primary source of protease in unripe papaya is from its latex produced by 2-3 months-old fruits. Therefore, the latex of unripe-papaya fruit and its senesced leaves have promising potential as a tenderizer for beef and mutton.

The effect of papain on meat structure is determined using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), which potentially describes the protein bands involved in meat tenderness, and also evaluates meat quality before and after papain treatments (Onopiuk et al. 2019). The process also describes the protein band of myosin heavy chain (MHC) and myosin light chain (MLC) (Gao et al. 2017). MHC and MLC are bonded by non-covalent bonds, which dissociate during electrophoresis (Vikhlyantsev and Podlubnaya 2017). However, they still play an essential role in the strength of contraction and are also related to the composition of protein fibers. Muscle fibers with MHC and MLC bands

are the primary energy source for muscles (Zapata et al. 2009). The destruction of protein in meat is confirmed with a scanning electron microscope (SEM), which helps to effectively observe meat tenderization (Soji and Chulayo 2020). Also, this method accurately depicts muscle fibers and connective tissue, and it helps to evaluate muscle microstructure changes by showing the meat surface before and after papaya protease treatment (Barekat and Soltanizadeh 2017; Jun-hui et al. 2020). Furthermore, SEM is used to document protein changes, connective tissue, endomysium structure, perimysium structure (Hu et al. 2018), Z lines degradation, I band breaks, and sarcolemma attachment to myofibrils (Kartika et al. 2019). Therefore, this study aims to analyze the effectiveness of papaya fruit-latex and its yellow-senesced leaves as a natural and organic tenderizer.

2. Materials and Methods

This is an experimental study, which used papaya fruit-latex and yellow-senesced leaves as a tenderizer for meat. The papain enzyme was extracted from the young papaya fruit and yellow-senesced leaves using a modified papain extraction method (Welde and Worku 2018; Hafid et al. 2020; Yu and Zhang 2020), and the enzyme was then used for activity analysis and tenderization of the meat. The sample meats were obtained from shank and round part of the animal such as the back of the leg due to high intensity of motion and density of the its muscle fibers and cells.

2.1. Papaya fruit and leaf latex preparation

Young papaya fruit on the tree was tapped in the morning with a skin incision to collect latex in a sterile tube. The latex obtained was dissolved in distilled water in of 1:1 (v:v) ratio, then mixed and centrifuged with 15000 rpm at 4 °C for 10 min. The supernatant was then filtered using Whatman® qualitative filter paper, Grade 1 (WHA1001325, Sigma Aldrich) and freeze-dried. Meanwhile, the papaya leaves were washed and dried using vacuum drying at 50 °C, then the dried fruit and leaves were crushed to powder form and filtered using a 100-mesh sieve. Finally, the latex and yellow senesced leaf powder were used to coat the meat.

2.2. Papain enzyme extraction from papaya fruit latex by dialysis technique

50mL of latex was mixed with 50 mL of sodium bisulfate in the ratio of 1:1 (v:v), and the mixture was homogenized using a magnetic stirrer. Meanwhile, 250 g of papaya leaves were washed, blended, and filtered using a vacuum machine. The fruit and leaf solution was then collected, mixed with 0.1 mL of EDTA with a ratio of 1:1 (v:v), and homogenized for the activation process. The solution produced was mixed with 70% ammonium sulfate in a ratio of 1:1 (v:v) to increase the protein's solubility in water. Subsequently, the solution was precipitated by adding 96% absolute ethanol in a ratio of 1:2. The supernatant was then separated from the precipitated pellets using a centrifuge

at a speed of 5000 rpm for 15 min and the process was repeated.

The pellets were then dialyzed using a cellophane membrane with a buffer of 500 mL of 50 M Tris-HCl overnight in a refrigerator at 4 °C. The dialysis result was placed in an oven at 50 °C and mashed to powder. Meanwhile, the dialysis buffer was changed twice to avoid saturation of the buffer solution with salt.

2.3. Total meat protein

50 g of beef and mutton were covered with yellow papaya leaf and latex powder at concentration levels of 0 g, 10 g, 15 g, and 20 g for 4 h, respectively. The treated meat was then cleaned, mashed, and 5 g was taken, including the control. Subsequently, the sample was mixed with 15 mL of PBS 1X at a pH of 7.4, placed in a conical tube, refrigerated for 60 min, centrifuged at 4 °C at 3000 rpm for 15 min, then separated from the supernatant and the precipitates.

The supernatant was used to measure the protein concentration of the sample using a spectrophotometer. Meanwhile, spectroscopic blanks were prepared using 200 µL of Bioradrogen dissolved in 800 µL of distilled water. The final mixture was then prepared by mixing 1,000 µL of tested-sample, 2 µL of meat sample, 789 µL of distilled water, and 200 µL of Biorad protein assay reagent together. Finally, the absorbance was read on a spectrophotometer with a wavelength of 595 nm.

2.4. Papain activity measurement using Barford analysis

As much as 0.10 mL of enzyme sample was mixed with 1.10 mL (1%) of 0.10 M Tris-HCl at pH of 8 containing 12 mM cysteine. The reaction started by incubating the sample at 37 °C for 20 min, and it was stopped by adding 1.8 mL of 5% TCA. Furthermore, the separation process was carried out by centrifuging the sample at 3000 rpm for 15 min, and the peptide uptake that dissolved in the supernatant was measured with a wavelength of 280 nm. One unit of caseinolytic activity was defined as the amount of enzyme required to produce an increase of 0.01 absorbance units per minute under the conditions required for the test.

2.5. Procedure for Kjeldahl analysis

A small meat sample treated with around 3 to 10 mL of HCl 0.01 M was transferred to a Kjeldahl flask and mixed with 1.9 g of K₂SO₄, 40 mg of HgO, and 2 mL of H₂SO₄. An adjustment was then made to the mixture using 15 mg of 0.1 mL H₂SO₄ per 10 mg of meat with a mass above 15 mg. Subsequently, a few grains of boiling stone was added, and the sample was boiled for approximately 1 to 1.5 hs until the liquid was clear. It was then cooled by adding water slowly and it was finally refrigerated. The content of the pumpkin was transferred to the distillation tool and the flask was washed and rinsed 5-6 times with 2 mL of water, which was transferred to the distillation area. 123 mL of Erlenmeyer, containing 5 mL of H₃BO₃

solution and 2-3 drops of the indicator, was placed in a flask, and 10 mL of NaOH-Na₂S₂O₃ solution was added. The solution obtained was distilled until 15 mL of distillate was obtained. The condenser was rinsed with water and Erlenmeyer, while the contents of Erlenmeyer were dissolved approximately to 50 mL, then titrated with 0.02 N HCl until the color changed to gray. Finally, the protein percentage was statistically analyzed using ANOVA with a significant level of $p = 0.05$.

2.6. SDS-PAGE analysis

SDS-PAGE was carried out with the method: 2 g of the samples under different treatment conditions were mixed with 18 mL of 5% SDS (w/v) at 85 °C. The homogenate obtained was then incubated at 85 °C in a water bath for 1 h to dissolve the protein. Subsequently, it was centrifuged at 8000 rpm for 5 min at room temperature to remove insoluble debris. The supernatant obtained was mixed in a ratio of 1:1 (v/v) with a sample buffer of 0.5 M Tris-HCl with a pH of 6.8, containing 4% SDS, 20% glycerol, and 10% β ME. The mixture was boiled for 3 min, and the samples (20 μ g of meat protein) were placed on a polyacrylamide gel, which contains 10% gel and 4% spill gel). Finally, the gel was stained with staining solution, which contains 0.02% Coomassie Brilliant Blue R-250 (w/v) on 50% (v/v) methanol, and 7.5% (v/v) acetic acid.

2.7. Scanning electron microscope

The microstructure of the sample was evaluated using a scanning electron microscope. The muscle specimens were initially cut with a thickness of 2-3 mm and then fixed using 2.5% glutaraldehyde in 0.2 M phosphate buffer with a pH of 7.2 for 2 h. Furthermore, the samples were rinsed for 1 h with distilled water before dehydration in ascending grades of ethanol, namely 50, 70, 80, 90, and 100% (v/v). The samples were then dried with a critical-point drying process using Balzer's mod CPD 030, Blazer Process Systems, Vaduz, and Liechtenstein with CO₂ as the transition fluid. The dry samples were mounted on a bronze stub and sputtered with gold (Sputter coater, SC7620, Polaron, UK). The specimens were finally observed with a scanning electron microscope (SEM) at 250 \times magnification at an acceleration voltage of 10 kV.

3. Results and Discussion

Papain (EC 3.4.22.2) is an endolytic cysteine protease enzyme present in papaya plants and it is extracted from the latex, fruit, leaves, and roots (Amri and Mamboya 2012; Fernández-Lucas et al. 2017). The identification process showed that the enzyme level in the fruit-latex was significantly higher than the senesced leaves, as shown in Table 1. Furthermore, the application of raw latex and senesced leaves extract on the meat produced a different concentration in the total of meat protein. The percentage of mutton protein increased after fruit latex treatment, which was higher than senesced-leaves. However, the beef protein was lower than mutton after being subjected

TABLE 1 Activity and concentration of papain and protein percentage after treatment

Treatment	Enzyme		Meat Protein (%)	
	Wave absorbance	Concentration (ng/ μ l)	Beef	Mutton
Control			32.95 \pm 0.20	38.13 \pm 0.24
Fruit latex	2,47	85.60 \pm 0.00 a	29.86 \pm 0.02	40.98 \pm 0.17
Senesced-leaves	0,692	5.05 \pm 0.00 b	27.10 \pm 0.13	29.19 \pm 0.32

Note: the letter (a and b) indicates a significant concentration level analyzed using one-way ANOVA with confidence level 95%, and significantly $p = 0.05$.

to the same treatment. Papain has protein hydrolyzing activities, which breaks proteins into short chains (Parkash et al. 2021). Furthermore, it has better activity in myofibrillar hydrolysis than derived bromelain (Jun-hui et al. 2020), ficin, aspergills, and bacillus (Meshram et al. 2018; Lukin 2020), and it also observed capable in collagen lysis (Calkins and Sullivan 2001).

The protein concentration in muscle is determined by the myofibril that composes the myocyte (Moczkowska et al. 2017). Papain is a protease destroys the cell and large-sized fibril structure, consequently, the protein length is reduced, and the mass molecules converted into the small amino-acid molecule (Abdeldaiem et al. 2019). The protein percentage in the control group was higher than the treatment, except fruit latex effect on mutton, as shown in Table 1. This finding indicates that papain in fruit-latex is more effective as a natural tenderizer for beef than mutton. However, the papain activity of senesced leaves is higher than fruit-latex as the enzyme actively degrades the leaf's organelles to regenerate essential nutri-

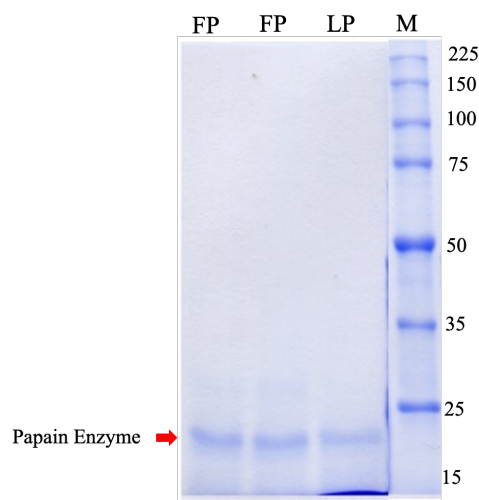


FIGURE 1 SDS PAGE papain from latex and senesced papaya leaves (FP: papain from fruit latex; LP: papain from senesced leaves; M: marker)

ents for young tissue development.

Pure papain from papaya fruit latex showed a molecular weight of 21 kDa and 21.3 kDa when purified using Sephadex (Monti et al. 2000). Based on SDS-PAGE visualization, the papain compound from fruit-latex and senesced leave has the same size, namely below 25 kDa, as shown in Figure 1. These findings are in line with the characteristics of the monomeric papain enzyme, which has a molecular weight of 23.4 kDa, and the maximum temperature for its activity of 37 °C. Furthermore, the enzyme has a hydrophobic core and is more hydrophilic in the outer part (Lambri et al. 2014).

Senesced-papaya leaves treatment decreases the thickness of protein band from various sizes, as shown in Figure 2. A high concentration of papain increases protein degradation, which is confirmed by the numerous short protein band observed in SDS-PAGE, and the thick band indicates a high protein concentration. Meanwhile, the small protein fragments observed in fruit-latex treatment were abundant as shown in Figure 2. A comparison of the visualization band of the SDS-PAGE indicates that papain effectively degrades protein in beef than mutton.

Protein change was observed in treated meat, and comparison using the SEM method showed few thick bands in the treated meat. This finding indicates that the long and dense protein loosens and shortens during papain treatment. Furthermore, the thinning of protein bands indicates a decrease in protein concentration at a specific molecular weight (Onopiuk et al. 2019), due to protein hydrolysis by papain. Papain is very effectiveness in degrading collagen and myofibril protein, resulting in smaller protein fragments of a specific size (Holyavka et al. 2019). Previous researches describe papain from leaf or unripe fruit strongly able to break muscle fiber structure of mutton (Is-trati 2008; Saeed et al. 2017; Ningrum et al. 2018) and beef (Barekat and Soltanizadeh 2017; Saeed et al. 2017; Ikram et al. 2021). In this research, myosin heavy chain was not detected, even though it should be observed in molecu-

lar weight at 140 kDa to 90 kDa. Contractile proteins, including actin and myosin are difficult to destroy using temperature (Gao et al. 2017; Ranatunga 2018) but they are destroyed using papain. Furthermore, intramuscular connective tissue also experience osmotic dehydration after treatment with papain (Gerelt et al. 2000) The observations using SEM showed that collagen fibers in mutton and goat have a complex structure that is neatly arranged and attached to the muscle fibers. Meanwhile, beef and mutton treated using senesced papaya leaves, and fruit latex had different microstructures from the control group. After enzymatic treatment, the collagen fibers were damaged, and their structure did not adhere to muscles. The collagen fibers also underwent denaturation, shrinkage, damage, irregularity, and noticeable gaps between muscle fibers (Barekat and Soltanizadeh 2017)

Although the papain concentration of senesced leaves observed in this study was lower than fruit latex, its activities in breaking protein bonds were still impressive, as shown in Figure 3. This was confirmed by the widening of the spaces between the muscle fibers, and the presence of visible erosion in the connective tissue (collagen). Meanwhile, the muscle fibers in meat treated with latex shrank and were separated from other muscle fibers. Muscle fibers in the control beef group were interconnected by the collagen. In contrast, beef treated with yellow papaya leaves has a wide space between muscle fibers than beef without treatment, as shown in Figure 3. The wide spacing of muscle fibers is caused by damaged connective tissue, while the mutton treated with papaya fruit latex had less collagen (connective tissue) than the control group, as shown in Figure 4. This loss of collagen led to the space between the meat fibers and the loosening of the meat.

The papain enzyme has an active site, comprising three amino acids, which act as the primary catalyst on site. Furthermore, the catalyst helps to break peptide bonds between C carboxylates and N amine of amino acids present on the protein backbone. Papain's catalytic activity leads

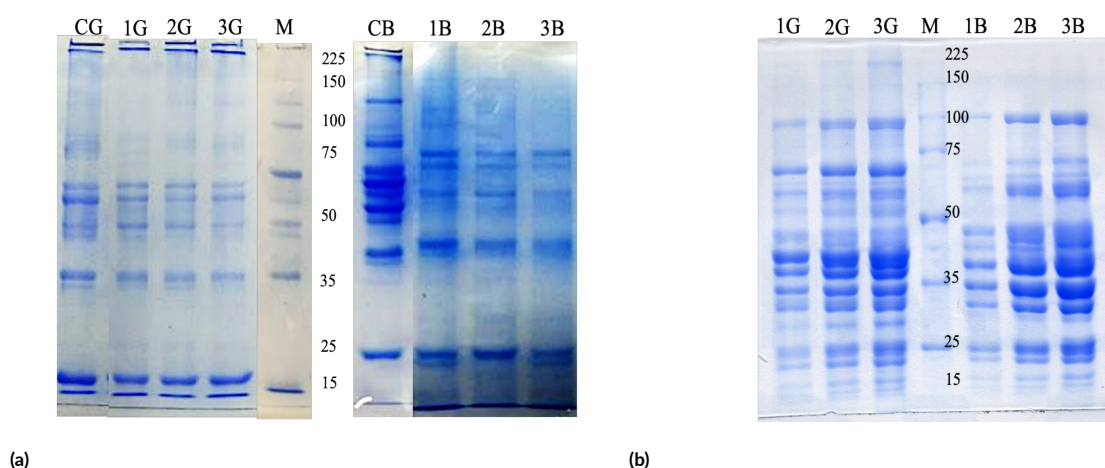


FIGURE 2 SDS PAGE of mutton and beef protein after treatment using papaya fruit latex (A) and senesced papaya leaves (B). Note: CG = control mutton with no treatment; 1G-3G = mutton with papain treatment 20 g,15 g, and 10 g, respectively; CB = control beef with no treatment, then 1B-3B = beef with papain treatment 20 g,15 g, and 10 g, respectively

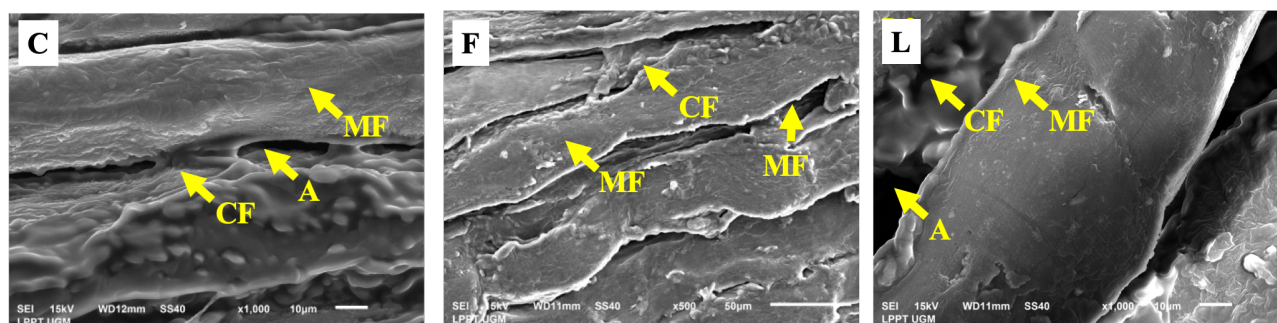


FIGURE 3 SEM photographs of collagen fibers of beef in control meat (C), papaya fruit latex powder (F), and leaves powder (L). CF = collagenous fibers; MF = muscle fiber; A = loosen collagen fiber bonds. The band was observed using 1000X magnification.

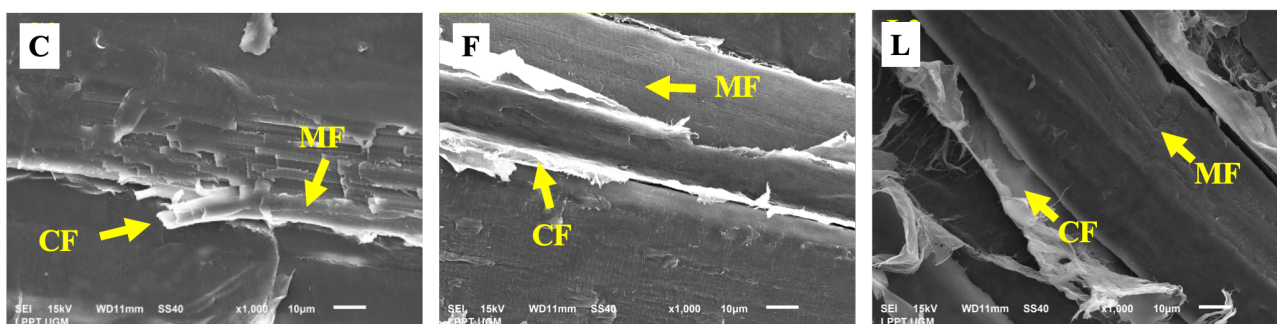


FIGURE 4 SEM photographs of collagen fibers of mutton in control meat (C); papaya fruit latex powder (F), and leaves powder (L). CF = collagenous fibers, MF = muscle fiber. The band was observed using 1000X magnification.

to the release of N-terminal groups, which causes the formation of smaller protein chains (Fernández-Lucas et al. 2017; Budama-Kilinc et al. 2018). However, the interaction of papain with meat causes more of the surface of the meat to come in contact with the enzyme's active site, which leads to massive protein breakdown. This breakdown led to the soft properties observed after the treatment with papain. The catalytic mechanism of papain is initiated by deprotonation of Cys-25 by the His-159 imidazole ring, which is facilitated by Asp-175 (Denessiouk et al. 2020). Although the positions of these three amino acids in the peptide sequence of the enzymes are far apart, folding causes the formation of the active side, which has a single function. Cys-25 performs a nucleophilic attack on the carboxylate carbon of the peptide backbone. Furthermore, Cys-25 and His -159 were considered catalytically active as thiolate-imidazolium ion pairs at the active site of papain (Holyavka et al. 2019).

The catalytic mechanism of papain revealed the decrease in protein count observed by the Barford test mechanism. The breakdown of the peptide bond in the main chain causes a change in the amino acid structure, which lose its amine group. However, the test did not detect the loss of these amines. The result of the Barford test shows a decrease in amino acid levels, which indicates the catalytic activity of papain as it destroys the main chain structure of the protein.

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Authors' contributions

This research was constructed by cooperating program includes: Conceptualization, A.I.K.; instrument and methodology, A.I.K. and H.S.K.; validation, S.D.; formal analysis, A.I.K. and H.S.K.; investigation, H.S.K.; resources, S.D.; data curation, A.I.K. and S.D.; writing—original draft preparation, A.I.K. and H.S.K.; writing—review and editing, S.D.; visualization, A.I.K.; supervision, A.I.K and S.D.; project administration, S.D.; funding acquisition, A.I.K. All authors have read and agreed to the published version of the manuscript.

Competing interests

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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