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Electrophoresis (SDS-PAGE) as a Method for Screening Species of Passiflora Using Seed Proteins as Molecular Markers

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Abstract: Brazil is the center of diversity and dispersion of species of the genus Passiflora. Two species of passion fruit *Passiflora tenuifila* BRS VT (passiflora garlic) and another of *Passiflora setacea* BRS PC were used in this study. The plants were grown on the Embrapa Cerrados experimental farm. The fruits were washed, minimally cut and their constituents separated. Both species differed in the proportion of wet mass between husk, seed, and pulp. As expected, both species had a higher proportion of husks and seeds and a lower amount of pulp. A semi-purification procedure for the proteins present in the seeds and husks was described and the molecular aspects were analyzed by polyacrylamide gel electrophoresis (SDS-PAGE). The quantification of soluble proteins was performed using the Bradford method. The analysis of the soluble protein extracted from the seeds and husks showed that these values are up to 70 times higher for the seeds of *P. tenuifila* and 28.5 times for *P. setacea*, in the husks. It was verified that both species present a similar protein profile, observed by the intense and diverse bands found in the polyacrylamide gel, mainly in the range of 32 to 19 kDa. This work opens up an unexplored field of tracking bioactive proteins and/or peptides including forms of nanostructure systems that protect other bioactive molecules. The objective of this work was to present an analytical procedure to semi-purify seed proteins of two Passiflora species and use electrophoresis as an analysis tool for further screening of their protein profiles and selection of a molecular marker to differentiate them.

Key words: Protein screening method, bioactive peptides, seeds, *P. setacea*, *P. tenuifila*, mass distribution on fruit.

1. Introduction

Passion is a fruit produced by plants of the Passiflora genus. The most commercialized in Brazil is the yellow species, named *Passiflora edulis*, also known as passion fruit. Brazil is the center of diversity and dispersion of species of the genus Passiflora. More than 150 wild species of passion fruit are described with the indication for utilization as food and medicinal purpose [1]. In 2008, Passitec Network started to improve sustainable use of the diverse passion fruit cultivated in Brazil by characterization of their functional/medicinal properties [2].

Based on the result of PASSITEC *Passiflora tenuifila* killip and *Passiflora setacea* decandole more promising properties are shown. The species named *P. tenuifila* BRS VT is in the market launch phase and *P. setacea* BRS PC, was launched by Embrapa in 2013 [1, 3]. Both species were genetically improved by PASSITEC for yielding rate.

Different stages of development, environmental stimuli, and stresses due to soil and climate conditions and even pest attacks can affect the expression of various proteins in plants. Therefore, studying a plant proteome can be more complex than studying an animal proteome of apparently similar size. Proteomics today can be divided into gel-based techniques: the most used methods in gel-based proteomics comprise the separation of proteins by sodium dodecyl sulfate polyacrylamide gel

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electrophoresis (SDS-PAGE). This can be performed in a single dimension based on molecular weight or in two dimensions also based on an isoelectric point of proteins in addition to molecular weight. Gel-free techniques generally comprise chromatography and tandem mass spectrometry [4]. Bioinformatics and also predictive methods that use secondary structure information and other protein properties have been complementary tools used in the attempt to identify these complex structures [5].

Plant proteins are always at the center of protein research in the nutritional field. In this sense, passion fruit has low protein content and cannot be considered a food for this nutritional purpose, as its protein value is in the range of 0.9% to 2% [6]. However, it has been described that this fruit (*Passiflora edulis*) has a lot of protein (13%) in the seed present inside [7]. This fact indicates that passion fruit seeds have the potential to be a source of bioactive peptides as concerning the high level of protein together with the application of enzymatic technology in the search for new peptide sequence. As a nutrient, it could be used as a complementary protein in food, and a protein of high nutritional quality as they could have essential amino acids of a food with genuine protein [7]. As a nutrient, passion fruit carries a protein of high nutritional value that is composed of essential amino acids in high concentration [7].

Thus, it should be considered that this part of the fruit can be used for molecular characterization of proteins and peptides. Both molecules have the potential to develop bioactive effects, either indirectly (obtained through enzymatic technology) or directly (naturally present in the seed). The first exemplification was applied by the use of enzymes for the hydrolysis of tomato seed proteins to produce a peptide with antioxidant effect [8]. As a complementary elucidation, natural occurring proteins and peptides present in Passiflora can be characterized by electrophoretic analysis. It has been described for *Passiflora edulis* values protein molecular mass in the range of 66 to 10 kDa [9].

The seeds in general have high oil content. This macromolecule needs to be removed from protein extracts as they are great interferers for a good SDS-PAGE electrophoretic analysis. Three different ways to obtain protein isolates from dried seed flours have been described in the literature: no degreasing [10] and degreasing with acetone [11] or degreasing with hexane [12]. In the need for better separation of the bands without the interference of fat molecules, acetone is a good choice due to its lower toxicity and greater polarity.

The peptides consist of 2 to 20 amino acids and show molecular mass of less than 6 kDa as bioactive molecules. They are resistant to peptidase, they absorbed by the intestine and transported through the bloodstream, and thus end up developing their physiological activities in the target tissues [13]. Some peptides can reduce hypertension, oxidative stress, show anti-cancer effect and have impact on Alzheimer's and Parkinson's [14-16]. The protein could be a transport system for other compounds as vitamins curcumin, resveratrol, caffeine, and quercetin [17]. Whether proteins present in passion fruits, especially the species improved by PASSITEC, have this kind of effect is not known yet. Therefore, the study of the proteins present in the passion fruit varieties, developed by the Passitec network, would help in the characterization of new natural nanostructured systems.

In this first screening work, a methodological adjustment is shown for the extraction and characterization of these proteins by SDS-PAGE electrophoresis, quantification of soluble proteins by the Bradford method, and physical analysis of the fruits regarding the proportion of seed mass, pulp, and peel on a wet basis are made. This study, therefore, intends to be the basis for future prospective work in proteomics and peptidomics for these two passion fruit varieties. Two areas that are still poorly studied

will be mitigated for them: bioactivity in degenerative diseases of the central nervous system and the formation of nanostructures that can carry other bioactive substances. Thus, the primary objective of this work was the presentation of an analytical procedure to screen the molecular weight of the main proteins and peptides of the seeds and peels samples of the two species of passion fruit studied.

2. Materials and Methods

2.1 Feedstock

Two species of passion fruit *Passiflora tenuifila* BRS VT (passiflora garlic) and another of *Passiflora setacea* BRS PC were used in the study. The plants were cultivated in the experimental farm of Embrapa Cerrados, 15°36′13.02″ S; 26 47°43′17.34″ W, an approximate altitude of 1,050 m, Planaltina, DF. The crops were established in a 3-strand trellis, containing 30 plants with spacing between plants in the *P. setacea* cultivation of 2.5 m and the *P. tenuifila* cultivation of 0.8 cm and 2.5 m between rows, with drip irrigation. The dimensions of the pits were 60 cm in diameter by 60 cm in depth, obtained with the aid of a drill bit. The planting fertilization was done with dolomitic limestone to raise V to 50%, P_2O_5 (Super Simple) 250 g/hole; N32 (ammonium sulfate) 100 g/pit; K_2O (potassium chloride) 100 g/well; FTE BR12 100 g/pit; 33 organic matter (poultry litter) 10 L/hole. The seedlings were transplanted into 34 holes at 3.5 months of age. All fruits produced in the first half of 2020 were kept frozen. From the harvest, 3 kg of fruits of each species were randomly separated. The Biochemistry Laboratory of Embrapa Agroindústria de Alimentos studied the physical analysis, preparation of extracts, quantification of soluble proteins, and analysis of proteins by SDS-PAGE electrophoresis.

2.2 Sample Preparation

The fruits were washed, chopped and their constituents separated into three parts: peel, seeds, and pulp. The quantification of the wet mass was performed by weighing the three parts of the fruits. The seeds were separated from the juice by filtration in cotton cloth. The juice was not used. A semi purification procedure for isolation of seeds and peels proteins was utilized with degreasing step of 3 washing with acetone of pro analysis (PA) grade. The seeds retained in the filter and the minimally processed peels were dried under ventilation at a temperature of 60 °C for 48 h. For protein extraction from the dehydrated seeds and peels, 30 g of the dried material were homogenized in a blender with 100 mL of distilled water. The crude extracts were filtered in cotton cloth, and the filtrate was used to quantify soluble proteins by the colorimetric Bradford method [18].

2.3 Preparation of Extracts for SDS-PAGE Electrophoresis

For electrophoresis analysis, the dehydrated seeds and peels (60 °C, 48 h) were ground in a blender and subjected to the degreasing process as described in Fig. 1.

Sample preparation for application in the electrophoresis gel was carried out by weighing 5 mg of defatted flour and solubilizing in 1 mL of electrophoresis buffer (4% sodium dodecyl sulfate (SDS), 12% glycerol, 2% mercaptoethanol, and 0.01% cromassie blue G250 dye at pH 6, 8) vortexed for 1 h.

2.4 SDS-PAGE Electrophoresis Analysis

In this study, the electrophoresis system of BIO-RAD Laboratories was used. The gel preparation methodology used was described by Laemmli [19]. For the running gel, acrylamide was used at a concentration of 12%, and in the application gel of the samples at a concentration of 4%. The run was carried out for 3 h under a voltage of 100 V in a gel of 10.8 × 6.8 cm. Six different proteins were used as molecular weight standard (in kDa): phosphorylase b (102.66); BSA (79.24); ovalbumin (46.38); carbonic anhydrase (33.87); soybean trypsin inhibitor.

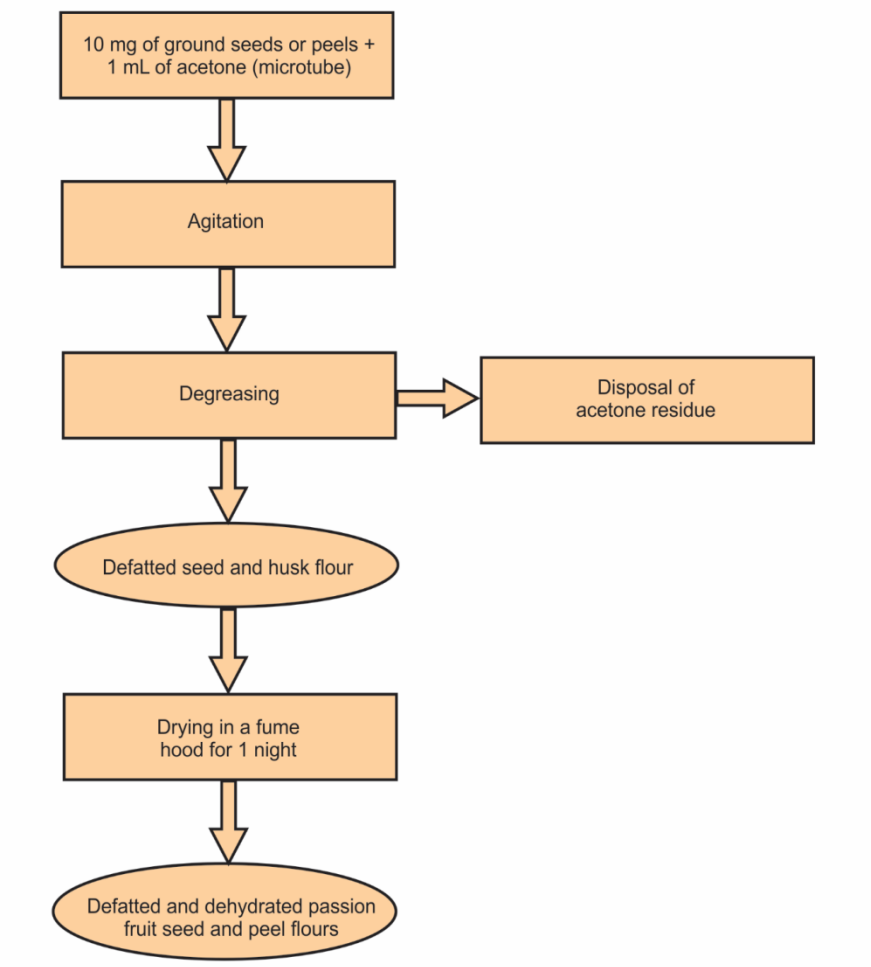

Fig. 1 Flowchart for obtaining defatted and dehydrated seed and husk flours.

3. Results and Discussion

The physical aspect of the species *Passiflora tenuifila* BRS VT and *Passiflora setacea* BRS PC shows a great distinction between them. The species *P. tenuifila* has yellow and wrinkled skin, due to the loss of water between harvest and storage (Fig. 2). The *P. setacea* has green skin with light green stripes without alterations, resembling a mini watermelon. No perceived signs of deformity due to possible dehydration between harvest and storage were observed (Fig. 3). Both species also differ in terms of the proportion of wet mass between the peel, seed, and pulp. *P. setacea* has a similar proportion between seed and peel mass from 3 kg of fruit. However, pulp mass is approximately 5 times smaller than the mass found in the peels and seeds (1:1:0.2). The wet mass of seeds and pulp is more balanced (1:0.97:0.8) (Table 1).

As expected, both species have a higher proportion of peels and seeds and a lower amount of pulp. Correa *et al*. [20] showed the importance of juice in the food industry and draw attention to the existence of the large volume of by-products generated.

The semi-purification method was important to improve the visualization of the bands of the proteins extracted from seed samples due to the interference of fat molecules in the electrophoretic separation. Another advantage is also due to the drying process that used low temperature/longer time to preserve the protein structures present in the matrix, in addition to the concentration effect.

The analysis of soluble protein extracted from the seeds and peels showed that these values are up to 70

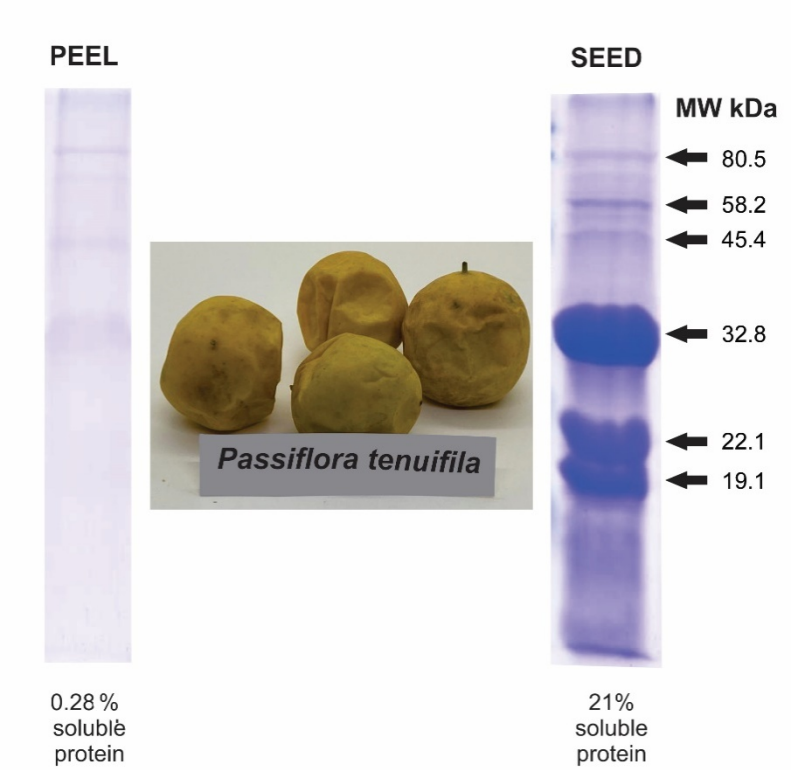

Fig. 2 Photo of *Passiflora tenuifila* BRS VF species, proportionality of protein soluble in peel and seed in 100 g of dry weight and protein profile obtained by SDS-PAGE electrophoresis.

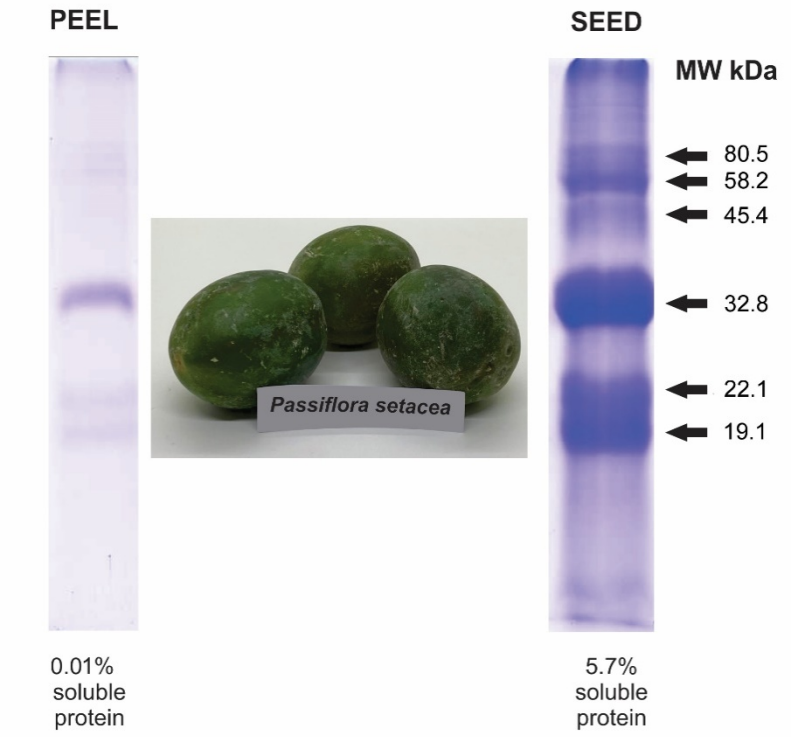

Fig. 3 Photo of *Passiflora setacea* BRS PC, proportionality of protein soluble in peel and seed in 100 g of dry weight and protein profile obtained by SDS-PAGE electrophoresis.

Table 1 Wet mass distribution (in g) of peel, seeds, and fruit pulp of the passion fruit species *P. setacea* and *P. tenuifila*.

Fruit part	*P. setacea*	*P. tenuifila*
Peel	1,238.1	609.1
Seed	1,239.2	592.2
Pulp	275.3	490.7
Total	2,752.6	1,692.0

Table 2 Content of soluble proteins (in %) present in the husks and seeds of the species *P. setacea* and *P. tenuifila* (up to 3 kDa).

Species	Soluble protein in seeds (%)	Soluble protein in the husks (%)
P. tenuifila	21.0	0.283
P. setacea	5.7	0.005

times higher for the seeds of *P. tenuifila* and 28.5 times for *P. setacea*, in relation to peels (Figs. 2 and 3 and Table 2). Therefore, the peels cannot be considered a source of protein. These biochemical-molecular results of seed proteins by electrophoresis indicate that *P. tenuifila* and *P. setacea* have some proteins or peptides of mass weight below 17.43 kDa (Figs. 2 and 3), that is the protein standard with lowest molecular mass used. The dark blue staining on the front of the gel emphasizes this conclusion. A molecule with low molecular weight (protein or peptide) is present in the method of electrophoresis SDS-PAGE [19]. These results are reinforced when the similarities and intensity of the protein bands are identified after the semi-purification method implemented in this work, see Fig. 1.

The molecular mass of proteins in the range of 85 kDa to 40 kDa present in seeds of the two species does not differentiate. Similarities between the two species are observed. However, the three bands in this range showed more intensity for extracts from *P. setacea*. It is known that the Bradford method of soluble protein quantification detects peptide chains up to 3 kDa which cannot be visualized by electrophoresis SDS-PAGE but detected by the method of Bradford (Table 2). Even so, it can be verified that both species have a lot of proteins, as observed by the intense and diverse bands found in the polyacrylamide gel (Figs. 2 and 3). These strongly stained bands can be observed in the range of 32 to 19 kDa.

Values of protein in order of 2.4%-2.8% were observed by Ramaiya *et al*. [21] in juice of passiflora. However, the method utilized was that of N-total that detects not only protein. These results allow us to suggest that this work is the start point of future studies of screening passiflora species having seed protein as molecular mark. Future work should be carried out with the protein extracted from seeds of these fruits, such as, aiming not only to prospecting new proteins for but also by the ability to form nanostructured systems [17].

Pelegrini *et al.* [9] described the presence of a peptide of 5 kDa in seeds of *P. edulis*. This peptide has an anti-fungal bioactive effect. So, another point of view is concerning evaluating peptides in the seeds. This study can be initiated with a scan made by TRIS-TRICINE electrophoresisas described by Stephan *et al*. [22]. This is an alternative technique in which occurs the substitution of the glycine buffer by tricine that allows low molecular weight proteins being more easily separated into a gel with a lower percentage of acrylamide [23].

As said, Holanda *et al*. [3, 24] quantified and confirmed the presence of alkaloids, anthocyanins, phenolic compounds and the absence of toxicity in *P. tenuifila*, but nothing was done regarding peptides and protein or even in the initial identification of peptides by electrophoresis, with the final objective to characterize these potential bioactives molecular, as recently described by Stephan [25]. So, these molecules are not characterized yet.

4. Conclusion

The semi-purification procedure of the proteins of the passion fruit seed *P. setacea* and *P. tenuifila* showed that almost all the protein of this fruit is present in the seed. A new procedure for the preparation of protein extract with a good degree of purification is shown using electrophoresis (SDS-PAGE) as a molecular analytical method. Although the protein extracts of these species studied have similar profiles, future studies with other species should be carried out to obtain a screening of protein markers in the *Passiflora* species.

Thus, this work shows a useful method to study Passiflora seed proteins to describe sources of new proteins that can stand out from a nutritional or techno-functional point of view.

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