Electrophoresis SDS-PAGE and Tricine-SDS-PAGE to differentiate black and yellow soybean: a molecular approach

DOI: 10.46932/sfjdv2n2-083

Received in: March 1st, 2021
Accepted in: May 30th, 2021

STEPHAN, M.P.
DSc-Biochemistry
Embrapa Agroindústria de Alimentos
Avenida Das Américas 29.501- Rio de Janeiro-RJ-Brasil CEP 23020-470

ABSTRACT

Soybean is well recognized for having several bioactive compounds. Black soybeans have been used in Eastern countries as a healthy food and as an medicinal alternative. The identification of natural substances, such as peptides, present in black and yellow soybeans is necessary. An evaluation of the contribution of these molecules to positive effects to human health molecular studies should be done. The search for molecular aspects that may explain this difference between black soybean and yellow soybean is extremely important. In order to have a peptide-protein extract with semi-purified aspects for further molecular characterization, soybeans grains were grounded in a home-use coffee grinder and fat was removed by using acetone. The protein extracts obtained were characterized by two eletrophorectic techniques (SDS-PAGE and Tricine-SDS-PAGE). Polyacrylamide gels with clear and well-defined polypeptide chains showed that both black and yellow soybean genotypes presented the peptide 5 kDa, while, the 4 kDa peptide was only found in black soybeans. This study, on molecular bases, showed that this method to obtain peptide-protein extracts was effective, to separate peptides in that region and could contribute to choose black or yellow soybean genotypes for clinical trials in evaluation of different health effects.

Keywords: new natural peptide, molecular characterization, electrophoresis.

1 INTRODUÇÃO

Black soybean (Glycine max (L.) merril soybeans with a black seed coat has been used as health food and oriental medicine for hundreds of years (Kim *et al.,* 2015).The consumption of this culture has been associated with the reduction of different diseases, such as obesity, cardiovascular diseases, diabetes, certain types of cancer and immunological disorders (Görgüç *et al.,* 2020). These effects, with therapeutic characteristics, have been attributed to proteins or even to the lunasin peptide that naturally occurs in this grain (Jang *et al*., 2008; Chatterjee *et al.*, 2018; Hao *et al.*, 2020).

The development of transgenic soybean with overexpression of the natural peptide Lunasin (5 kDa) is a recent genetic engineering performed with yellow soybean (Hao *et al.*, 2020). In this work, Hao *et al.* (2020) proved by in vitro assay the anti-cancer and anti-inflammatory effect of this modified grain and proposed its utilization as functional food.

The potential of soybean as a supplier of these peptides comes from the high content of nitrogen molecules in its constitution. Soybean has high amount of protein (35-40%) and this fraction has the the globulins, β conglicin and glycinin as the major molecule content. Moreover, its satisfactory levels of essential amino acids indicate that this seed is a good matrix for either gastrointestinal digestion or enzymatic processing with production of bioactive peptide (PBA) (Chatterjee *et al*., 2018; Sánchez and Vásquez, 2017).

Bioactive peptides can be 2 to 20 amino acids chain length and are absorbed and carried by blood circulation through intestinal absorption, in order to develop their physiological activities in the target tissues, being also resistant to digestive peptidases (Kitts and Weiler, 2003). As described by *Görgüç et al*. 2020 the biological effect of peptides vary according to the type, sequence and molecular weight of amino acids. These molecules may also have hormonal like actuation as described by Wang and De Mejia (2006) and Chatterjee *et al*. (2018), in peptides obtained by innovative technological processes. The anti-obesity action of some black soybean PBAs has been described with studies using "*ïn vitro*" assay (cellular models) and "*in vivo*" tests (adults aged 19 to 65 years), respectively (Kim *et al*., 2007; Kwak, 2012).

The authors show a leptin (peptide hormone) in soy peptides. It should be noted that leptin is a hormone that decreases the absorption of fat in human cells and consequentially has an anti-obesity effect. The scientific literature has shown data on molecular characterization of hydrolysis of yellow soybean protein isolates by using electrophoresis in SDS-PAGE (Mora-Escobedo *et al*., 2009). However, this electrophoretic technique does not allow the visualization of peptides in the range of 10 kDa and 1 kDa. The TRIS-Tricine/SDS-PAGE electrophoretic technique has been described by Schagger and Jagow (1987) and can be used for detection of mass peptides up to 1 kDa.

The applicability of this technique in the area of Food Science and Technology is well described on studies of protein hydrolysis during cheese maturation (Carneiro *et al*., 2019) and heat processed meat (Stephan *et al*., 2013). This paper will show a new methodological strategy for extracting peptides of soybean with the aim of comparing the electrophoretic peptide pattern between the yellow and black cultivar. These results will help to choose the best soybean genotype to be used for clinical studies of anti-obesity effect in obese individuals.

2 MATERIAL AND METHODS

Thirteen different lines of black soybean and 13 black soybean 7 genotypes of yellow soybean were used. Some genotypes have no lipoxygenase and this description is described in Table1 and 2. Two different types of electrophoresis were performed, SDS-PAGE 12% (Laemmli, 1970) and Tricine-SDS-PAGE (Schägger and Jagow, 1987) to identify polypeptide chain bands of the twenty three lines mentioned above. The grains were grounded in a home-use coffee grinder (Cusinart) and kept at room temperature until utilization. One gram of each flour was submitted to the degreasing process using 10 mL of acetone, under agitation for 1 hour. The upper two thirds of acetone containing the fat present in the flours were removed using pasteur pipette and the lower third was transferred to an eppendorf centrifuge and submitted rotation rate of 12,045g in a centrifuge during 5 minutes. The precipitates obtained after centrifugation were left in the eppendorf tube itself, during a night in a gas exhaust chamber for drying and evaporation of acetone. Defatted protein extracts with the mass of 3 or 5 mg were solubilized in 1 mL of the sample buffer (pH 6.8 with 4 % SDS, 12 % glycerol, 2 % mercaptoethanol and 0.01 % Coomassie blue G250) and applied to the gel (20µL). Two types of electrophoresis were used: SDS-PAGE and Tricine SDS-PAGE

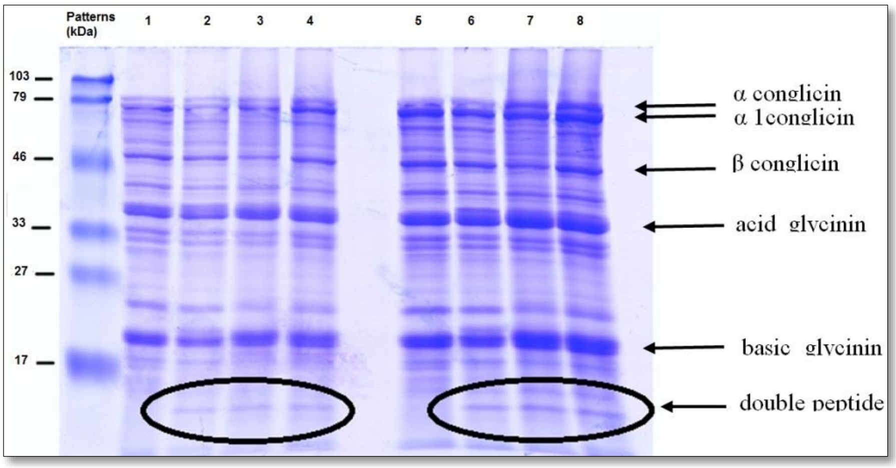
3 RESULTS AND DISCUSSION

The Figure 1 shows the protein/peptide electrophoretic pattern of four cultivars of soybean (one from yellow and three from black genotypes) obtained by SDS-PAGE 12% (Laemmli, 1970). The innovative method of sample preparation described in material and methods used to remove fat from the grounded soybean grains was effective. The protein extracts obtained after defatting process were prepared in the concentration 3 and 5 mg/ml and applied to polyacrylamide gels of SDS-PAGE). A gel without spread staining and with well-separated polypeptide chains was obtained. The concentration of 5mg/ml was gave the best staining intensity. The subunits of globulins 7S (β conglycin) and 11S (glycinin), major soybean proteins present in soybean grain, can be observed in SDS-PAGE polyacrylamide gels.

As expected, protein patterns of the black and yellow soybean, respectively, BRM09-50901 and PF122105 were similar in the molecular mass range between 100 and 17 kDa. However, as concern to peptide pattern (bands below the position of 17 kDa standard) a differentiated fingerprint between black and yellow soybean was observed. However, no calculation of the peptide could be done because SDS-PAGE protein standards do not migrate in the range below 17kDa (Laemmli, 1970). Initially, three well- known black soybeans (the well-known genotype soybean BRM09-50901 and one two others from retail trades) and the genotype yellow genotype PF122105 were used. Surprisingly, double peptide bands were observed only for black beans. For the yellow genotype PF122105, only a single peptide band was detected. Between the two concentrations used, the concentration of 5mg/ mL showed better bands with more intense staining.

For better elucidation of lower molecular weight peptide, some modifications were realized: one related to analytical technique (Tricine-SDS-PAGE instead of SDS-PAGE) and other by including more genotypes of black (13) and yellow soybean (7). For this purpose, the utilization this Tricine-SDS-PAGE electrophoretic technique that allows the identification of peptides up to 1kDa (Schägger and Jagow, 1987) was chosen. This methodology has been used in the study of the degree of hydrolysis in other food products, whether in the maturation of cheese (Carneiro *et al*., 2019) or heat treatment of meat products (Stephan *et al*., 2013)

Figure 1: Soybean electrophoretic (SDS-PAGE 12%) protein-peptide pattern in two extracts concentrations: 3 e 5 mg/mL. 1-5- yellow soybean (PF 122105) 2-6- black soybean (BRM09-50901) 3-4-6-7-black soybean from retail trade

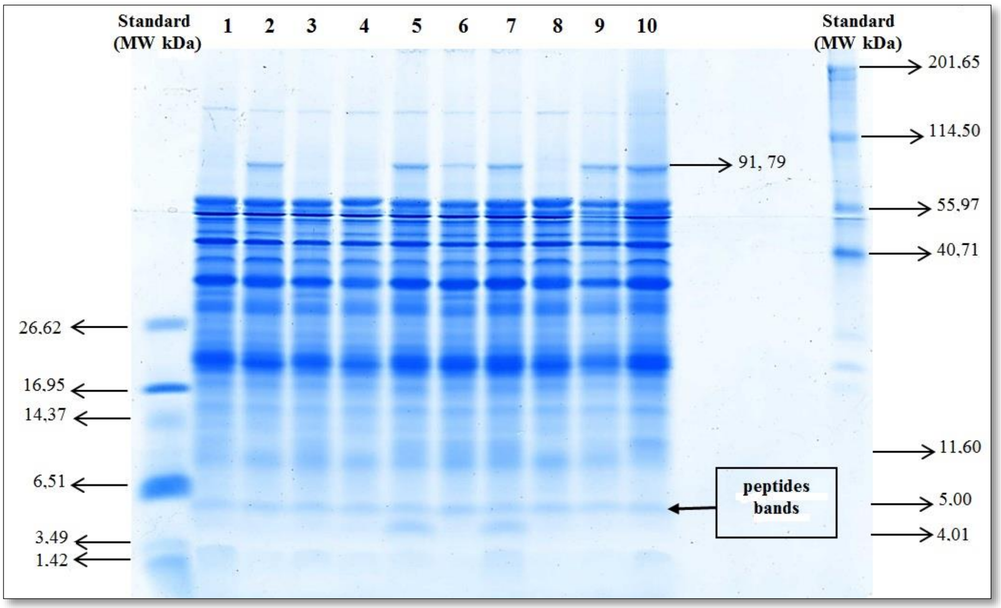


In Figure 2, only one yellow soybean variety was used, six new genotypes were tested for yellow soybean (VMAX; BR267; BR157; PF12210; YELLOW PEEL) and two for black soybean (PFAH1758; GENOTYPE OF ANTI-OBESITY PROJECT). Similar results as that obtained in SDS-PAGE can be observed by the absence of 4kDa peptide in all yellow soybeans. However, among the three black soybeans evaluated in this same gel, only two of then presented confirm the results obtained in SDS-PAGE (BRM09; GENOTYPE OF ANTI-OBESITY PROJECT). The double peptides bands was observed only in black soybean showed molecular mass of 4kDa e 5 kDa

This work has as future prospects to choose the best soybean genotype to be used for clinical study with obese patients, so, two purposes must be attended: a) right choice of black and yellow genotypes b) adequate taste diets formulations to be prepared with genotypes with the absence of lipoxygenase (LOX-).

A natural peptide called Lunasin of 5 kDa (Lule *et al*., 2015) has been described to occur in yellow soybean (Hao *et al.,* 2020) and this peptide can be visualized in Figure 2, both for yellow and black soybean. However, among the three black soybean genotypes tested, only two of them presented this new peptide of 4 kDa, as observed Lanes 5 (BRM09-50995, LOX+) and 7 (anti-obesity project, LOX+). As the presence of this peptide was not observed in one of the three black soybean genotypes studied, more 13 black soybean genotypes were used for a more comprehensive evaluation (Figure 3). The Figure 2 and Table 1 clearly detail these characteristics for yellow soybean genotype and Figure 3 and Table 2 for black soybean. The anti-obesity action of some black soybean PBAs has been described with studies using "*ïn vitro*" assay (cellular models) and "*in vivo*" tests (adults aged 19 to 65 anos), respectively, (KIM et sl., 2007) and KWAK *et al.,* 2012). Of the thirteen genotypes tested, only six presented the 4kDa peptide.

Figure 2: Soybean electrophoretic (Tricine-SDS-PAGE) protein-peptide profiles iof seven different lines of soybean: seven yellow and three black. Protein extracts utilized in the concentration of 5mg/mL. 1- VMAX (YELLOW, LOX+) 2- BRS267 (YELLOW, LOX+) 3- BR157 (YELLOW, LOX-) 4- PF122105 (YELLOW, LOX-) 5- BRM09-50901 (BLACK, LOX+) 6- PFAH1758 (BLACK, LOX-) 7- BLACK (anti-obesity project, LOX+) 8-YELLOW (anti-obesity project, LOX-) 9- YELLOW (anti-obesity project, LOX+) 10- YELLOW (retail trade- casa pedro)



years), respectively (Kim *et al*., 2007; Kwak, 2012) but none of this studies have been focused in molecular analysis, specially using electrophoresis (SDS-PAGE) and (Tricine-SDS-PAGE) . It should be emphasized that these results show data not yet described in the literature which may be useful both for the choice of genotype for clinical trials and for future proteomic assays.

Nine genotypes (BRM095095, BRM 10043, KOJE PRETO, LATE GIANT, WILSON BLACK, Z4Z4K9, PFAH14111, PFAH19266, PF133086) has lipoxygenase in its constitution and area called LOX+, but only five of these nine genotypes the presence of 4kDa peptide (BRM095095, BRM 10043, KOJE PRETO, PFAH19266, PF13306), Figure 3 Table 2. It can be concluded that this in this paper it is suggest is a new natural peptide presents in some genotypes of black soybean. A natural peptide of 5 kDa has already been describe for soybean (Lule *et al.*, 2015). In three genotypes (PFAH17158, PFAH19268, PFAH19267) the absence of lipoxygenase and the 4 kDa peptide were observed. Five genotypes showed similarities of yellow soybean, no 4kDa peptide and the presence of lipoxygenase. Only one genotype (PFAH184) was LOX- and has the 4 kDa peptide. It can be concluded that only the genotype PFAH184 is indicated for future clinical studies (LOX- , 4 kDa peptide). The use of it will allow getting a diet with attractive taste (LOX-), as well as the evaluation of the actuation of double peptide in the anti-obesity effects. "by Rho *et al.* (2007) described a correlation anti-obesity effects of black peptides in obese rats.

Figure 3: Electrophoretic (Tricine-SDS-PAGE) protein-peptide profile of thirteen different genotypes of black soybeans. Protein extracts utilized in the concentration of 5mg/mL 1-BRM09-50995, 2- BRM10-043, 3- koje Preto, 4- Late Giant, 5- Wilson Black, 6- Z4Z4K9, 7- PFAH 14111, 8-PFAH 17158, 9- PFAH18184, 10- PFAH19266 9-

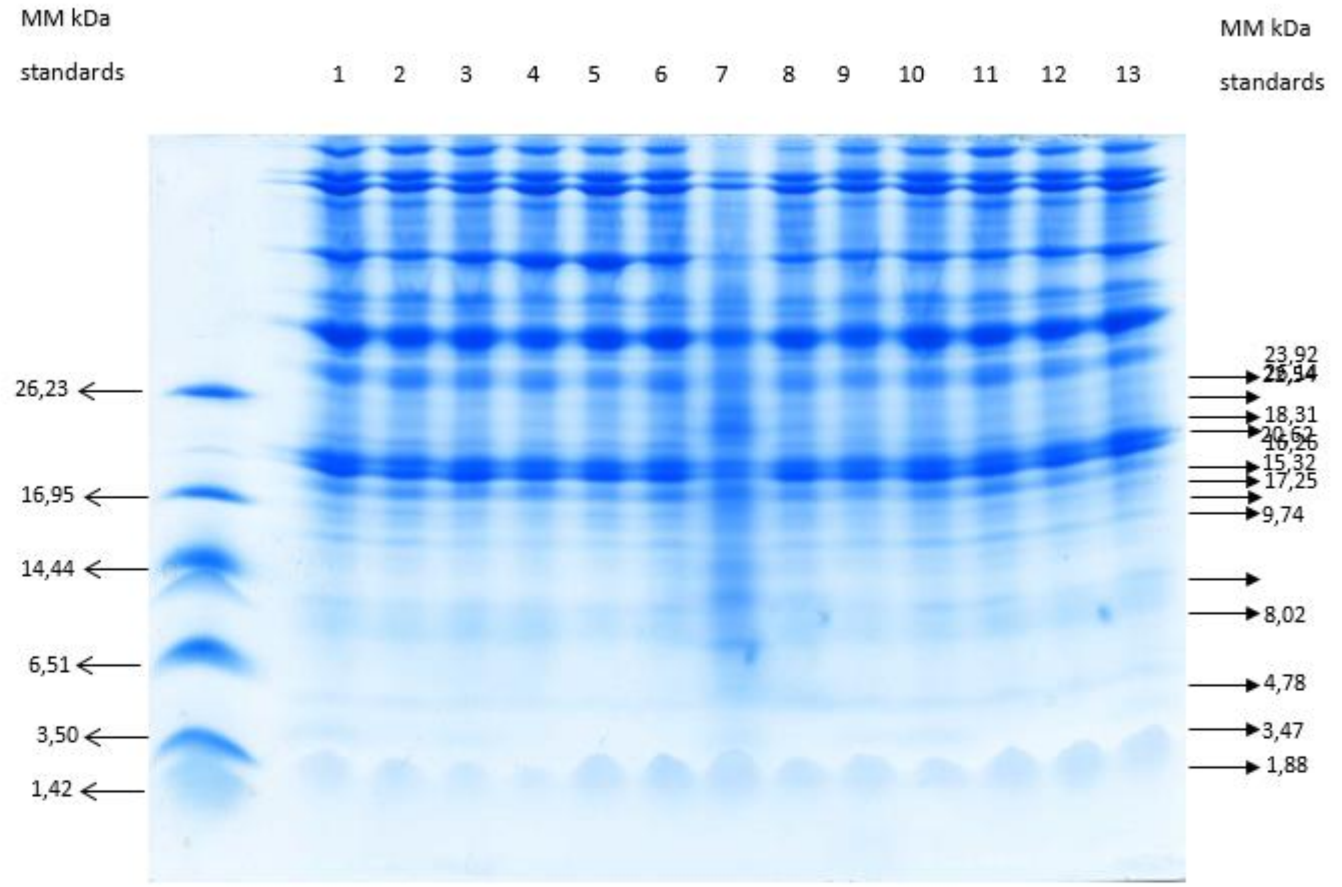


Table 1: Evaluation of the presence of 4 kDa peptide (Figure 2) in different black and yellow soybeans genotypes and their characteristics related to the presence of lipoxygenase

Genotypes of soybean	lipoxygenase Presence (+) or absence(-)	peptide presence (+) or absence(+)
VMAX (yellow)	(+)	(-)
BRS267 (yellow)	(+)	(-)
BR 157 (yellow)	(-)	(-)
PF122105 (yellow)	(-)	(-)
BRM09 (black)	(+)	(+)
PFAH17158 (black)	(-)	(-)
anti-obesity project (black)	(+)	(+)
anti-obesity project (yellow)	(-)	(-)
anti-obesity project peel (yellow)	(+)	(-)
trial trade (casa pedro- Yellow)	(+)	(-)

Table 2: Evaluation of the presence of 4 kDa peptide (Figure 3) in different black soybean genotypes and their characteristics related to the presence of lipoxygenase

Genotypes of black soybeans	lipoxygenase presence (+) or absence(-)	peptide presence (+) or absence(+)
BRM09	(+)	(+)
BRM 10-043	(+)	(+)
KOJE PRETO	(+)	(+)
LATE GIANT	(+)	(-)

WILSON BLACK	(+)	(-)
Z4Z4K9	(+)	(-)
PFAH14111	(+)	(-)
PFAH17158	(-)	(-)
PFAH18184	(-)	(+)
PFAH19266	(+)	(+)
PFAH19267	(-)	(-)
PFAH19268	(-)	(-)
PF133086	(+)	(+)

4 CONCLUSION

The search of peptides that may have potential for biological activity in black and yellow soybean was performed. The method developed for purification of samples to obtain cleaner protein-peptide extracts associated with electrophoresis technique SDS-PAGE and Tricine-SDS-PAGE proved to be effective. Polyacrylamide gels were clear and with well-defined polypeptide chains for both yellow and black soybean. Very promising data were obtained after critical analysis of the photo-documented data. The search for peptides in black soybean and yellow soybean showed that only black soybean genotypes present two peptides (4 and 5 kDa). These results serve as a molecular basis for future choice of soybean genotype with potential to be used for clinical trial studies and for proteomic analysis.

ACKNOLEGMENT

Sincere thanks to the technical assistant of Alessandro Araújo dos Santos and the analyst Tatiana de Lima Azevedo, respectively, for the contribution in all stages of the analytical process of the work (since sample preparation until electrophoretic analyses) and for data organization and peptides molecular weight calculation.

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