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Structural changes in the proteins from two species of the genus vigna by effect of different treatments

Cambios estructurales en las proteínas de dos especies del género vigna por efecto de diferentes tratamientos

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Abstract

A study of the thermal treatment effect on the protein structure of the rice bean and cowpea bean was conduced. The protein was isolated by isoelectric focusing and electrophoretic pattern, FT-IR and FT-Raman spectroscopy were used for their characterization. The results showed that in both species the thermal treatment originate modifications on the conformational structure modifying the secondary structure, disorder level and agregation fractions. The FT-IR and FT-Raman spectros indicate that the main secondary structure is the protein of both species, was the β-sheet with a smaller contribution of the α-helix structure. The structure 3_{10} helix and the sulfhydril groups were detected in the protein of both species, in addition the presence of some aminoacids also were observed (Cys, Lys, Trp, Phe, Tyr and Met). The electrophoretic pattern showed a significantl reduction in the number of high molecular weight subunits by effect of thermal treatment and isolation process, two hight molecular weight bands are mantained before and after treatments (23 kDa and 50 kDa), these fractions could be a stable subunits and common ancestor in both species.

Keywords: *Vigna unguiculata*, *Vigna umbellata*, IR, Raman, spectroscopic.

Resumen

Se realizó el análisis del efecto del tratamiento térmico sobre la estructura de la proteína del frijol arroz y frijol vaquita, durante su aislamiento. La proteína fue aislada por medio de precipitación isoeléctrica, y se caracterizó por medio de espectroscopía FT-IR y FT-Raman, así como el análisis de su perfil electroforético. Los resultados indican que en ambas especies, los tratamientos utilizados provocan cambios conformacionales de la proteína, generando desorden, desenrrollamiento o agregación de su estructura secundaria, lo cual se ve reflejado en los espectros de FT-IR y FT-Raman, donde la estructura lámina-β se presentó como mayoritaria con menor contribución de la estructura α-hélice, encontrando la estructura hélice 3_{10} tanto en el aislado del frijol arroz como en el hidrolizado del frijol vaquita, así mismo se pudo detectar la presencia de los grupos sulfhidrilo y algunos aminoácidos (Cys, Lys, Trp, Phe, Tyr and Met). El patrón electroforético mostró pérdida de las subunidades de alto peso molecular por efecto del tratamiento térmico y aislamiento, adicionalmente en ambas especies se observaron dos bandas con pesos moleculares de 23 kDa y 50 kDa, las cuales se mantienen aún después de los diferentes tratamientos, indicando su estabilidad.

Palabras clave: *Vigna unguiculata*, *Vigna umbellata*, IR, raman, espectroscopía.

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1 Introduction

The genus Vigna includes around 80 species which only 7 species have been domesticated, 5 in the Asian region and 2 in the African region (Iseki *et al*., 2016). *Vigna umbellata* and *Vigna unguiculata* (rice and cowpea beans) are two domesticated species, however, both species are underutilized crop pulses.

The rice bean is cultivated principally in the Eastern and North-Eastern regions of India and consumed as cooked vegetables (tender pods and green shelled seeds). The nutritional value of the rice bean is high, reports indicate protein content between 16 and 19 % that include essential amino acids such as methionine (0.52-0.67 g/16 g N) and tryptophan (0.85-2.42 g/16 g N). Also, the rice bean is rich in carbohydrates (56-59 %), fiber (6-7.5 %), thiamine, riboflavin, niacin, iron, phosphorus and calcium with high bio-availability (59.8 %) (Saikia *et al*., 1999; Katoch, 2011; Hoque *et al*., 2016; Kujur *et al*., 2017). The flavonoids, the total and individual phenolic contents, like so the antioxidant and antidiabetic compounds of rice bean indicate that this legume could have a high nutraceutical potential (Yao *et al*., 2012).

On the other hand, the cowpea bean is cultivated in Asia, Africa, South Europe and Central and South America. In this legume, the seeds are the main part consumed as food; however the leaves, fresh peas and fresh pods also consumed as food. The cowpea bean, contain between 18 and 25 % of protein content that include essential amino acids as lysine, histidine, isoleucine, leucine, phenylalanine and threonine, high carbohydrates content (50 to 67 %), high level of unsaturated fatty acids, potassium, calcium, zinc and iron (Goncalves *et al*., 2016; Devi *et al*., 2015). In addition, several reports have indicated that the bioactive compounds of cowpea bean could to prevent some chronic diseases such as cardiovascular disease and some types of cancer (Ojwang *et al*., 2015).

Have been reported that both legumes (rice and cowpea beans) contain several compounds that could affect the bioavailability of their nutrients, such as phosphorus, polyphenols, saponins, trypsin inhibitors, phytic acid, tannins, α-amylase inhibitors, and flatulence producing saccharides (Saikia *et al*., 1999; Hoque *et al*., 2016) enzyme inhibitors (trypsin and chymotrypsin principally), hemagglutinins, cyanogenic glucosides and oxalic acid (Goncalves *et al*., 2016). For this reason, several processing methods have been used to reduce the level of antinutritional factors such as, hydrothermal treatments, separation of components (concentrates and isolates) and modification of compounds (hydrolysates) (Kaur and Kawatra, 2000; Saharan and Khetarpaul, 2001; Devi *et al*., 2015). However, all treatments used for to reduce the antinutritional factors originate the loss of another compounds as both soluble protein and thermolabile protein (Saikia *et al*., 1999; Marrugo-Ligardo *et al*., 2016) and to originate modifications on the structural conformation of the proteins; these last modifications could be analyzed by the vibrational spectroscopy (FT-IR and Raman), because this spectroscopic methods provide chemical and structural information that help understand modifications on the macromolecules (Devi *et al*., 2015; Güler *et al*., 2016).

The spectroscopic methods such FT-IR and FT-Raman have been used to determine modifications on the major food components. The associated changes to the modifications of the structure are mostly connected to bending and skeletal vibrations in the FT-IR and FT-Raman spectroscopy. Structure of food proteins have been analyzed using FT-IR and FT-Raman spectroscopy, these techniques consider several different and conformationally sensitive vibrational modes on -CO-NH- amide or peptide bonds, with the amide I and III bands as principal components used for the secondary structure (Kong and Yu, 2007). Recently, several researches have been conducted using FT-IR and FT-Raman focusing to determine modifications on the secondary structure of proteins by effect of different treatments such as proteolysis process, thermal treatment, high electric field, cold-pressure and isoelectric precipitation. The findings on modifications of the conformational structure of the proteins have been reported principally about the secondary structure as the transitional effect of α-helix structure to the β-sheet structure during protein gelation, unfolding of proteins, interactions between CO2 and specific amino acids and generation of disordered structures and random coil (Meersman *et al*., 2002; Kong and Yu, 2007; Rygula *et al*., 2013; Vanga *et al*., 2016; Güler *et al*., 2016; Kobayashi *et al*., 2017; López *et al*., 2018; Zhang *et al*., 2018). On the other hand, FT-Raman spectroscopy has been used in addition to FT-IR spectroscopy to determine the cysteine sulfhydryl (SH) groups in proteins (Raso *et al*., 2001).

However, few studies have reported on the effect of several modification processes (thermal treatment, isolation and hydrolysis) using electrophorectic pattern, FT-IR and Raman spectroscopy to determine modifications in the structure proteins. For this reason,

the goal of this study was the use of both FT-IR and FT-Raman spectroscopy in addition to Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) to determine the effects that the treatments have on the structure of proteins of two Vigna species.

2 Materials and methods

2.1 Vegetal material

Two species of Vigna genus, rice bean (*Vigna umbellata*) and cowpea bean (*Vigna unguiculata*) were obtained from Zacatlan Puebla, Mexico. The seeds were selected, any extra material was removed and then the beans were grounded by a domestic coffee grinder. The dried bean powder was packed in PVC bags, labeling as RBP (Rice Bean Powder without thermal treatment) and CBP (Cowpea Bean Powder without thermal treatment), and stored in LG Model GR-452SH refrigerator (LG electronics, Mexico) at 4ºC until used.

2.2 Hydrothermal treatment

The rice and cowpea beans were dispersed in distilled water (10% w/w) under agitation for 1 h at 20 ºC (Eberbach Corporation, Ann Arbor, MI, USA) at 200 rpm, boiled in a water bath at 90ºC for 25 min, and stored overnight in a freezer at -40ºC (Thermo-Revco, Modelo ULT2186-5-A31, Asheville, North Carolina). The bean samples were subsequently freeze-dried in a freeze dryer (Freezone Plus 6, Labconco, USA) at -40ºC and 0.30 mPa until constant weight (approximately 72 h) and ground with a domestic coffee grinder (Hamilton, Modelo 80365, Hamilton Beach, USA). The dried rice and cowpea beans powder was finally sealed in an airtight container, labeled as RBT (Rice bean with termal treatment) and CBT (Cowpea beans with termal treatment) and stored at 4ºC until used.

2.3 Preparation of protein isolates

2.3.1 Isoelectric point

Protein isolates from rice bean and cowpea bean powders were obtained via isoelectric precipitation as described by Bernardino-Nicanor *et al*. (2000). The isoelectric point of the beans proteins was determined as the pH value of maximal precipitation. The meal:water ratio was 1:20, the pH of the suspension (9 to 11) was kept constant during the extracting by adjusting with 0.1N NaOH. The temperature (40 to 50ºC) was regulated by a water bath. After 30 min, the slurry was centrifugated (Hermle Z200A, Germany) at 6000 rpm for 30 min, the supernatant was collected and the pH was adjusted at range 3 to 7 with HCl (0.1N), the protein precipitate was separated by centrifugation at 6000 rpm for 30 min and freeze dried (Freezone Plus 6, Labconco, USA).

The isolates were obtained from the beans with and without thermal treatment and labeling as; IRB (protein Isolate from Rice Bean without thermal treatment), IRBT (protein Isolate from Rice Bean with thermal treatment), ICB (protein Isolate from Cowpea Bean without thermal treatment), and ICBT (protein Isolate from Cowpea Bean with thermal treatment).

2.3.2 Protein content determination

The protein content was determined according to the method Kjeldahl (AOAC, 1995), using the 6.25 factor.

2.4 Enzymatic hydrolysis

The protein isolates were hydrolyzed by sequential treatment with pepsin (P7012, Sigma) and pancreatina (P1750, Sigma), according to the method of Mora-Escobedo *et al*. (2009). The protein isolate was suspended in distilled water to prepare the protein substrate (44 mg/mL). The protein solution was adjusted by the addition of 1N of HCl to pH of 2 and a temperature of 37ºC for 60 min. A measured amount of pre-suspended pepsin in distilled water and adjusted to hydrolysis pH conditions was then added to the substrate in order to obtain an enzyme/protein ratio of approximately 4.5% (AU/w). Later, the solution was adjusted by the addition of 0.9 M of $NaHCO_3$ to a pH of 5.3, then a solution of pancreatin, with an enzyme/protein ratio approximately 4.5% (AU/w) was added, the mix was gently homogenized and then the pH was adjusted to 7.5 using 1N of NaOH and a temperature of 37ºC; the reaction mixture was maintained for 120 min. The hydrolysis reaction was stopped by heat treatment of the reaction mixture to 90ºC for 10 min. All procedures were carried out in a 100 mL glass reactor.

The samples were labeled as; HRB (Hidrolysate of the protein isolate from Rice Bean without thermal treatment), HCB ((Hidrolysate of the protein isolate from Cowpea Bean without thermal treatment), HRBT (Hidrolysate of the protein isolate from Rice Bean

with thertmal treatment) and HCBT (Hidrolysate of the protein isolate from Cowpea Bean with thermal treatment)

2.4.1 Determination of the degree of hydrolysis (DH)

The degree of hydrolysis was obtained as protein solubility in trichloroacetic acid (TCA), according to Kim *et al*. (1990). An aliquot of 10 mL of hydrolysate was solubilized in 10% TCA solution, and after 15 min, centrifuged at 6000 rpm for 15 min. The nitrogen content of the hydrolysate and the supernatant of the sample treated with TCA were analyzed by Kjeldahl method (AOAC, 1995). The calculation of the degree of hydrolysis (DH) was conduced as follows.

$$DH(\%) = \frac{Soluble\ N_2\ in TCA\ (10\%)}{Total\ N_2\ in\ the\ sample} \times 100 \qquad (1)$$

were DH is degree of hydrolysis; N_2 is nitrogen; TCA is trichloroacetic acid.

2.5 FT-IR spectroscopy

The FT-IR spectra of the rice bean and cowpea bean (meal, isolate and hydrolysates without and with thermal treatment) were acquired on a Perkin Elmer FT-IR spectrophotometer (Perkin Elmer, Inc., MA, USA) using potassium bromide (KBr) discs prepared from powered samples mixed with dry KBr. The spectra were recorded (16 scans) in transparent mode at a resolution of 4000-400 cm^{-1}.

2.6 FT-Raman spectroscopy

The Raman measurements were performed in a Perkin-Elmer (Perkin Elmer, Inc., MA, USA) 2000R NIR FT-Raman Spectrometer equipped with an Nd:YAG laser emitting at a wavelength of 1064 nm and an InGaAs detector. For these analyses, 180º backscattering refractive geometry was used. The spectrometer was managed using Perkin-Elmer Spectrum software (Spectrum^{TM}). The spectral data for rice beans and cowpea beans (meal, isolate and hydrolysates without and with thermal treatment) were obtained at a wavenumber resolution of 4 cm^{-1} at a nominal laser power of 500 mW. For each spectrum, 20 scans were accumulated to ensure an acceptable signal-to-noise ratio. All Raman spectra were collected at room temperature.

2.7 Polyacrylamide gel electrophoresis

SDS-polyacrilamyde gel electrophoresis (SDS-PAGE) was performed according to the Laemmli method (1070), using the Mini Protean 3 Cell (Bio-Rad Laboratories, Hercules, CA 94547 USA) vertical unit. Molecular masses of the polypeptides were calculated using the following standard proteins (Bio-Rad Laboratories, Hercules, CA 94547 USA): phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), α-lactalbumin (14.4 kDa). Protein samples were dissolved in sample buffer (0.1 mol/L Tris-HCl, pH 6.8, 20 mL/100 mL glycerol, 2 g/100 mL SDS, and 0.05 g/100 mL bromophenol blue). Gels were fixed and stained with Coomasie Brillant Blue.

2.8 Statistical analysis

The quantitative data were expressed as the mean ± standard deviation, and analysis of variance (ANOVA) was carried out, followed by Tukey´s test, with a confidence interval of 95% and $p \leq 0.05$. The software program Statistical Analysis System (SAS Institute Inc., Cary, NC, USA) v. 8.0 was employed for the data analysis, and all experimental determinations were assayed in triplicate.

3 Results and discussion

3.1 Protein content

The higher protein content was observed in the rice bean (30.4 ±0.10 % dry basis) in comparison with the cowpea bean (21.58 ±0.14 % dry basis), while that the higher moisture content was obtained from the cow pea beans (13.5 %), these results are in accordance with those previously published by Katoch, 2011, 2013; Kaur, 2000; Kaur *et al*., 2013 and Saikia, 1999. After thermal treatment the protein content in both species RBT and CBY was reduced 1.57 and 2.68, respectively. This phenomenon is likely due to the water-soluble proteins and nitrogenous compounds loss during the thermal treatment, in addition to the soaking process before thermal treatment (Rehman and Shah, 2005; Arcan and Yemenicioğlu, 2007).

3.2 Protein isolate yield

A variation of 0.5 was observed in the pH value at the isoelectric point calculated between RBP and CBP (4.0 and 4.5 respectively), according with previous report, these variations in the pH value of the isoelectric point could be due to the amino acid composition and the organization of the side chain in the 3D structure of the protein (Alexov, 2004). With the isoelectric point calculated, the protein yield of both Vigna species is shown in Table 2; apparently, the heat treatment decreased the protein content in both Vigna IRB and ICB species (25 and 0.45% respectively). The variations observed by the thermal treatment effect, according to some authors could be due to a solubility loss, originated by the self-cross-linking or self-aggregation of protein, protein unfolding and exposure of hydrophobic groups (Kharlamova *et al*., 2016; Zhang *et al*., 2018; Morais *et al*., 2019).

The results obtained in the present study are in agreement with reported by other autors that indicate that isoelectric point of the protein in legumes vary in the range of the acid pH values (Jayasena *et al*., 2011; Klupšaite and Juodeikiene, 2015).

3.3 Degree of hydrolysis (DH)

The hydrolysis degree of the both Vigna protein isolates (cowpea bean and rice bean) are shown in Table 1. A variation of around 10 % on the hydrolysis degree between the two species was observed, the highest hydrolysis degree was obtained from the HRB. Apparently, the variations observed could be attributed to the differences on the amino acid composition due principally to the specie, since variations on the amino acids composition between several species within the same genus and even within same specie have been reported (Domínguez-Perles *et al*., 2016; Baptista *et al*., 2016). Another reason for the hydrolysis degree variations have been reported by Chabano *et al*., (2007) who demonstrated that a compact and globular conformation of the proteins decrease their degree of hydrolysis.

According to other authors, different varieties or species present different protein structures, which endow proteins with different characteristics (Yang *et al*., 2016) and apparently this could be the cause in the differences of the degree of hydrolysis (10 %) observed between both two Vigna species tested. Few studies focused on the secondary structure of the Vigna protein has been reported and they have indicated that in the cowpea bean, the β-sheets secondary structure is the predominant (Mune and Sogi, 2016).

A higher effect on the DH between both two Vigna species by effect of the thermal treatment (16 %) than by effect of the specie (10 %) was observed. The HCBT showed a higher DH (value) than the HRBT (value) and according to other authors, the thermal treatments generate several reactions that modify the protein structure and this modification of the structure could generate different level of dissociation into proteins generating, several subunits, unfolding of their quaternary, tertiary and secondary structure, generation of more reactive sites, interactions via hydrogen bonding between functional groups exposed and generation of new disulfide bonds (Pan *et al*., 2005, Güler *et al*., 2016).

Table 1. Protein content and degree of hydrolysis of the isolates from rice and cowpea beans

	Protein content (%)	Degree of hydrolysis (DH) (%)
IRB	81.66±0.26^{a}	89.46±0.46^{a}
IRBT	61.25±0.19^{c}	63.83±0.33^{d}
ICB	72.05±0.16^{b}	80.22±0.75^{b}
ICBT	71.72±0.06^{b}	76.19±0.54^{c}

Values followed by different letters in the same column are significantly different ($p \leq 0.05$). The values are means ± SD of triplicate measurements. IRB (protein Isolate from Rice Bean without thermal theatment), IRBT (protein Isolate from Rice Bean with thermal treatment), ICB (protein Isolate from Cowpea Bean without thermal theatment) and ICBT (protein Isolate from Cowpea Bean with thermal treatment)

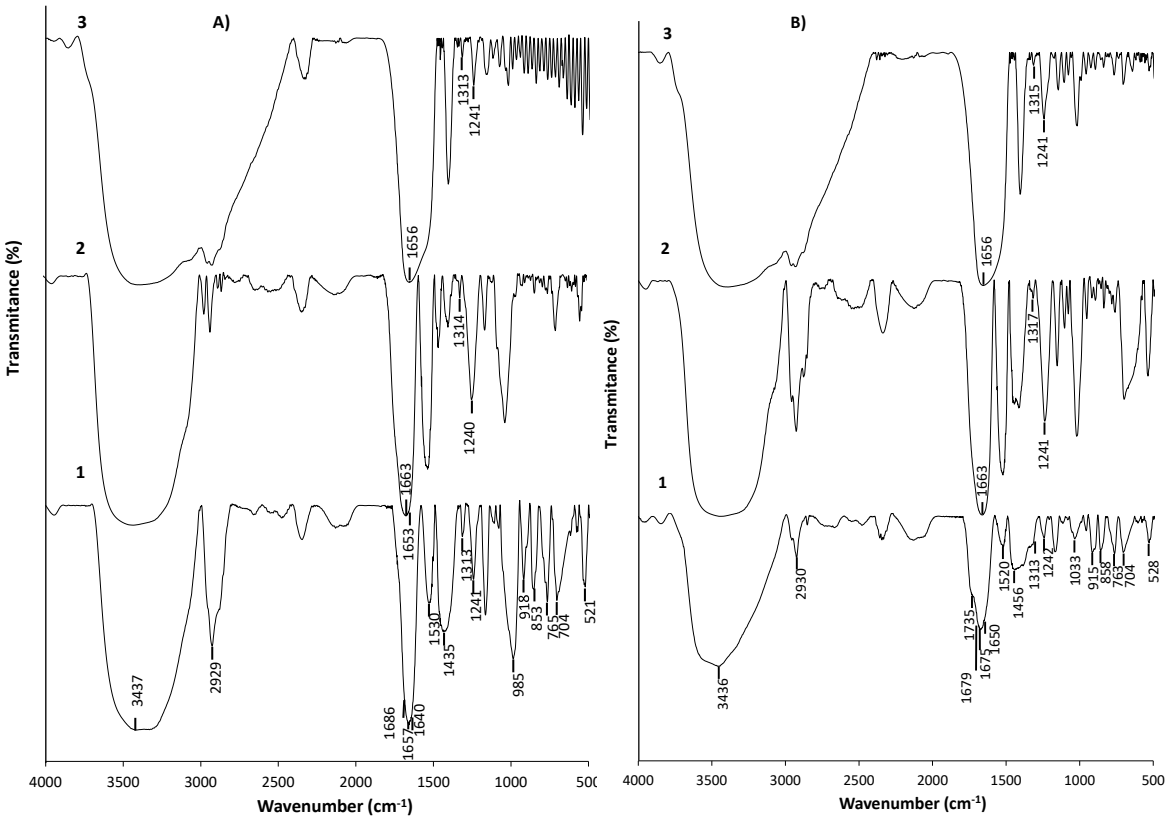

Figure 1. FT-IR patterns of the rice bean without and with thermal treatment. A) without thermal treatment, B) with thermal treatment. (1) Powder (2) Isolate; (3)Hydrolysate.

The DH obtained of both two *Vigna* species, rice bean and cowpea bean (Table 1) after thermal treatment are according to results reported for soy protein hydrolysates (73.5 %), and ayocote beans hydrolysates (73 %), obtained with the same enzymatic system (Mora-Escobedo *et al*., 2009; Teniente-Martínez *et al*., 2019).

The high DH obtained to both species, according to Tavano (2013) is due to the characteristics from the ezymes used, the trypsin is an endopeptidase which has preferential release of N-terminal Arg and Lys, and pepsin also an endopeptidase that has a preferential cleavage of amino acids hydrophobics, preferable aromatic residues, in addittion, previous studies reported that the DH is dependent not only on the enzymes used, but also on the treatment conditions, in this work the time of 180 min was used expecting structural and functional changes of the protein (Wang *et al*., 2008, Martínez-Palma *et al*., 2015; Fajardo-Espinoza *et al*., 2020).

3.4 FT-IR analysis of structural changes

In the FT-IR spectra of all samples tested, the bands characteristic of proteins were observed (Figures 1 and 2). However, highlighted two regions, the Amide I (1600-1700 cm^{-1}) and the Amide II (1500-1600 cm^{-1}) regions, which according to several authors, are the most sensitive and useful for the interpretation of the secondary structures of proteins (Meersman *et al*., 2002; Kong and Yu, 2007; Vanga *et al*., 2016).

Changes in the amide I region of the both species (Figures 1 and 2) were observed, due to the secundary protein structure changes, this region is mainly related to the C=O stretching vibrations coupled with the in-plane NH bending and influences the hydrogen bond patterns according to Meersman *et al*., (2002) and Baltacioğlu *et al*., (2015).

Figures 1A and 2A show the FT-IR spectra of the RBP and CBP. The bands located at 1657 cm^{-1} in the RBP spectra and 1656 cm^{-1} in CBP spectra, are assigned to α-helix structure due to the C=O

stretching vibration of the backbone chain. While, the characteristic band for β-sheets could be assigned to the band located at 1640 cm^{-1} to both specie, according to Meersman *et al*., (2002), Güler *et al*., (2016) and Vanga *et al*., (2016).

The band located at 1657 cm^{-1} of the RBP spectrum was dimished and relocalited at 1650 cm^{-1} in RBT (Figure 1B), this could be due the reorganization and partial loss of the original secondary structure, due to the unfolding of the α-helix structure, which is accompanied by aggregation process, by the thermal treatment effect according several authors (Damaschun *et al*., 2000; Dong *et al*., 2000; Meersman *et al*., 2002; López *et al*., 2018). The aggregation is reflected in the formation and growth of two new bands located at 1675 cm^{-1} and 1679 cm^{-1}, which are characteristic for intermolecular antiparallel β-sheet aggregation.

In the FT-IR spectra of the CBP (Figure 2B), a band located around 1656 cm^{-1} was observed and according to some authors this band have been associated to the protein α-helix structure (Kong and Yu, 2007; Dong *et al*., (2000), on the other hand, the bands around 1640 cm^{-1} localized in the FT-IR spectra correspond to protein β-sheet structure (Meersman *et al*., 2002; Dong *et al*., 2000), while the band around 1649 cm^{-1} corresponds to the protein unordered structure (Dong *et al*., 1994). The thermal treatment apparently causes a decrease in the unordered structure of the protein and was reflected by the loss of the band around 1649 cm^{-1} in the FT-IR spectra of the CBT. Some authors have attributed this phenomenon to the inter-molecular bonding and structural rearrangement of the protein during the thermal treatment generating new and stronger intermolecular interaction among their components caused by new hydrogen and disulphide bonds. Similar modifications generated by effect of other treatments have been described by other authors (Jin *et al*., (2020).

The isolation process induced the formation of the 3_{10} helix structure in the IRB and IRBT (Figures 1 and 2), which is clearly evident by the band located at 1663 cm^{-1}, characteristic band for the 3_{10} helix structure according to Vanga *et al*., (2016), which appears due to the pH changes used in the isolation process. The 3_{10} helix structure in the ICB and ICBT was not observed, this suggests that proteins from cowpea bean are more resistent to the pH changes and thermal treatment. These changes in the protein isolates from the rice bean and cowpea bean may result from differences in functional groups, amino acid composition and interactions among them (López *et al*., 2018).

The 3_{10} helix structure disappears in the HRB and HRBT (Figure 1) while the 3_{10} helix structure is found in the HCB (Figure 2). On the other hand, the bands located at 1240 cm^{-1} and 1242 cm^{-1} (Figures 1 and 2) observed in all samples of both species, correspond to β-sheet structure, while the bands located at 1313 cm^{-1} and 1317 cm^{-1} were assigned to α-helix according to López *et al*., (2018).

The amide II region of the RBP and CBP (Figures 1A and 2A), which showed a prominent band at 1530 cm^{-1} and 1541 cm^{-1} respectively, corresponds to the bending vibrations of N-H groups and stretching vibrations of C-N groups. These bands disappeared in RBT, CBT, IRB, ICB, IRBT and ICBT, and appeared bands at 1520 cm^{-1} to samples of the rice bean (Figure 1) and 1530 cm^{-1} to samples of the cowpea bean (Figure 2), which may be attributed to the gradual unfolding of protein structure when extraction at higher alkali pH was performed, according to Ignjatović *et al*., (2001); Meersman *et al*., (2002) and Kobayashi *et al*., (2017).

Figures 1B and 2B show the FT-IR spectra of the RBT and CBT, the bands at 1735 cm^{-1} attributed to stretching vibration of lipids according to Kobayashi *et al*., (2017), this band disappeared after the isolation and hydrolysis processes. These results indicated that chemical denaturation ocurred during both processes and that lipids were removed.

The bands in the spectral region (amide II) from all samples of both species ubicated at 1405 cm^{-1} and 1456 cm^{-1} (Figures 1B and 2B), are attributable to the antisymmetric and symmetric stretching modes of free carboxylate anions (Güler *et al*., 2016).

The spectral region at 2920 cm^{-1} and 2937 cm^{-1} in all samples of both species corresponds to asymmetric stretching vibration of CH_2 group (Striolo *et al*., 2003; Dogan *et al*., 2007), which is present in the native structure of both species, gradually decreases during the thermal treatment, isolation and hydrolysis processes. Other amide vibrational bands were affected by the thermal treatment, isolation and hydrolysis of the protein, mostly in the spectral region at 500 cm^{-1} and 1000 cm^{-1}, corresponding to region amide IV associated with OCN bending, amide V corresponds to out of plane NH bending and amide VI is associated to out of plane C=O bending.

In the amide A region of the all samples of both species no changes were observed, in Figures 1 and 2 the strong band at 3309-3400 cm^{-1} arises mainly from the NH stretching mode of proteins with contributions of the O-H stretching vibrations ocurring

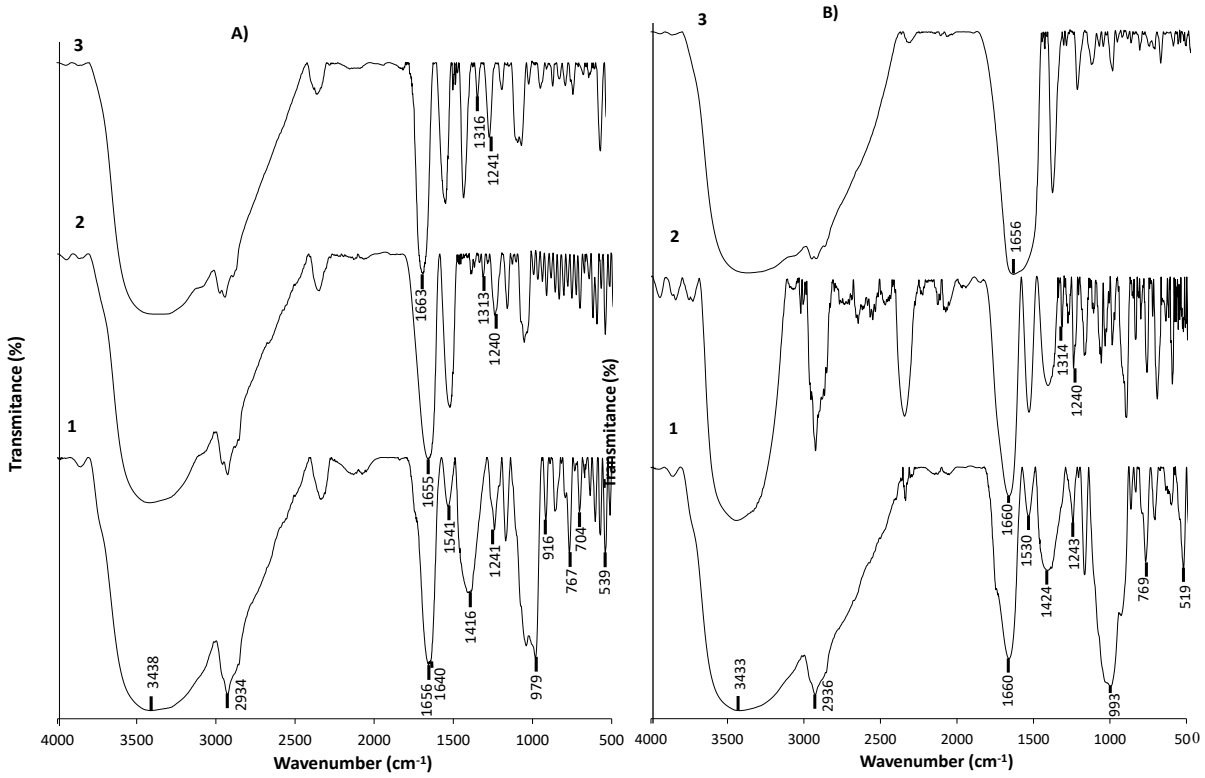

Figure 2. FT-IR patterns of the cowpea bean without and with thermal treatment. A) without thermal treatment, B) with thermal treatment. (1) Powder; (2) Isolate; (3) Hydrolysate.

in the hydrogen bonds and intermolecular H bonding (Dogan *et al*., 2007).

Variations in the FT-IR spectra of both species were observed, according to several authors, these variations could be due to amino acid composition, interaction between amino acids and the organization of the gruops into the 3D structure of protein (Kudre *et al*., 2013; Alexov, (2004).

3.5 FT-Raman spectroscopy analysis of structural changes

FT-IR and FT-Raman spectroscopy are complementary techniques that may be important to understand the structural changes of proteins during different process conditions.

Characteristic bands of the proteins in the FT-Raman spectra of both species were observed (Figures 3 and 4), in the regions of wavenumbers at 300 to 1800 cm^{-1} and 2800 to 3600 cm^{-1} (Raso *et al*., 2001).

The amide I bands (1645-1685 cm^{-1}) were mainly C=O stretching vibration in addition to N-H vibration, C-C-N vibration and C-N stretching vibration, while amide III region (1220-1350 cm^{-1}) were associated to C-N stretching vibration and the N-H in plane vibration of the peptide bond (Figures 3B and 4B). In addition, also bands around 2342 cm^{-1} and 2370 cm^{-1} that correspond to the NH stretching were observed (Kobayashi *et al*., 2017, Rygula *et al*., 2013).

The FT-Raman spectra of the RBP (Figure 3A), only showed the characteristic peaks of the amide III region (1268 cm^{-1} and 1269 cm^{-1}), which have been associated to the β-sheet structure; while that in the FT-Raman spectra of the CBP (Figure 4A), bands around 1679 cm^{-1} were mainly observed and according to some authors these bands are associated to the α-helix structure (Kobayashi *et al*., 2017, Rygula *et al*., 2013) and in a lesser extent, band associated to the β-sheet structure (1302 cm^{-1}). This finding is important due to the little research that indicate differences in the secondary structure of the protein between several Vigna species.

After thermal treatment in both Vigna samples in the FT-Raman spectra bands new bands or modification in observed bands in the Vigna without thermal treatment were obtained (Figures 3 and 4).

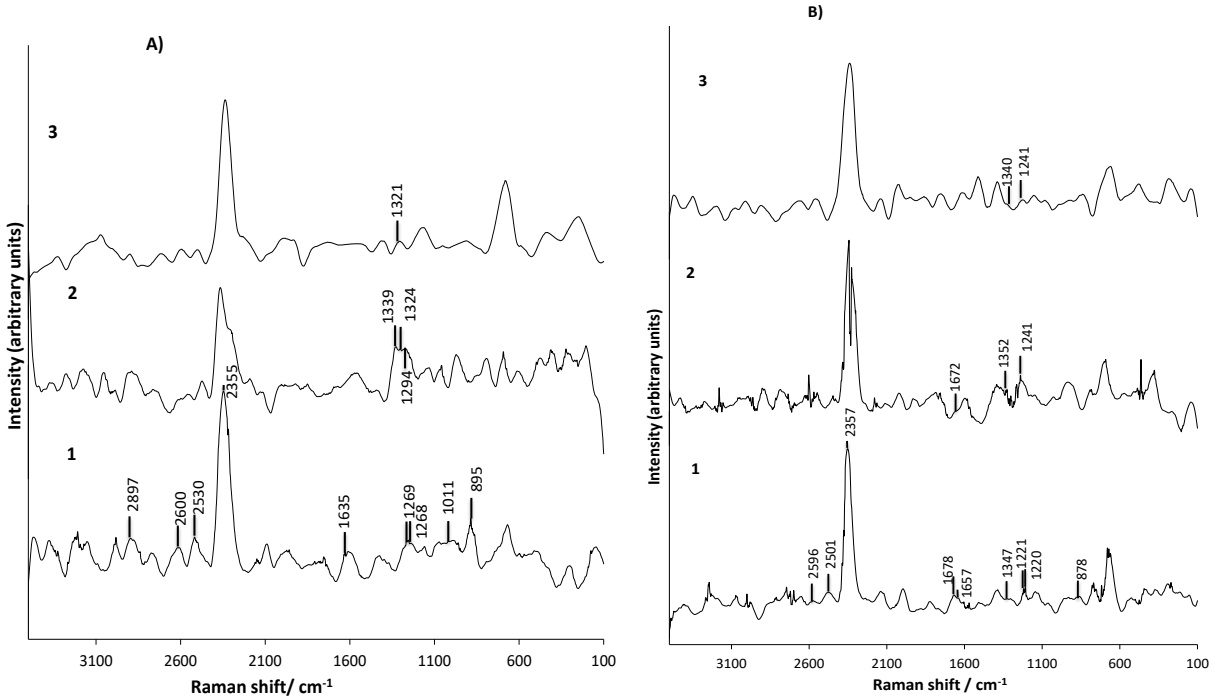

Figure 3. The Raman spectra of the rice bean without and with thermal treatment, A) without thermal treatment, B) with thermal treatment. (1) Powder; (2) Isolate; (3) Hydrolysate.

In the samples of the Vigna species after thermal treatment, bands associated to the α-helix structure were observed (1657 cm^{-1} and 1678 cm^{-1} to RBT, at 1672 cm^{-1} in IRBT, at 1679 cm^{-1} in CBP and at 1645 cm^{-1} in CBT spectras) Kobayashi *et al*., 2017, Rygula *et al*., 2013). In both Vigna samples thermally treated, the bands related to the β-sheet structure were maintained. These results could be due to changes in the protein structure during the thermal treatment, isolation and Hydrolysis according to Berhe *et al*., (2014), Kobayashi *et al*., (2017) and Hernández-Castillo *et al*., (2020).

On the other hand, bands between the 2500 cm^{-1} and 2600 cm^{-1} were observed and according with some authors, correspond to the S-H bonds of the proteins, apparently in both Vigna species the bands in the range of 2500 cm^{-1} and 2600 cm^{-1} indicate the presence of cysteine residues (Raso *et al*., 2001).

The band located at 2530 cm^{-1} in the FT-Raman spectra of the RBP (Figure 3A1), was assigned to the Cysteine-613, the bands at 2575 cm^{-1} and 2576 cm^{-1} in the FT-Raman spectra of the IRB (Figure 3A2) IRBT and HRBT (Figures 3B2 and 3B3), respectively were assigned to the Cysteine-635. The band at 2550 cm^{-1} in the FT-Raman spectra of the IRBT (Figure 3B2), corresponding to the Cystein-267, Cystein-287 and Cystein-458 with strong spectral contribution and hydrogen-bond strengths of cysteinde sulfhydryl groups of the protein.

In general, the IR-Raman spectra of all samples with some treatment, showed a decrease in the intensity of the bands (Figures 3 and 4) and according with other authors different processes could affect tertiary structure of the proteins, principally temperature and pH upper 70ºC and 10, respectively and apparently in both Vigna species, the thermal treatment and isolation conditions used in this work (90 ºC and pH 11 respectively) are the main responsible of the structural modification in the protein.

FT-Raman spectra of the cowpea bean showed not bands corresponding to the cystein residues indicated by Raso *et al*. (2001), however, bands ubicated in the range of 2518 cm^{-1} to 2600 cm^{-1}, suggest the cystein residues presence.

In both Vigna species, several bands that correspond to different amino acids (Table 2) were observed. The differences in number, localization and intensity of the bands in the FT-Raman spectra between the two Vigna species, apparently is due to the different treatments used as thermal treatment, isolation and hydrolysis process that

Table 2. Raman bands of the rice bean and cowpea bean corresponding to amino acids

Sample-Band (cm^{-1})	Amino acid
RBP-1011; RBT- 878, 1347; IRBT-1262, 1577; CBT-1586; ICB-1347	Tryptophan (ring stretching)
RBP-895	Lysine (C-C and C-N stretching)
IRB-1073; HRB-1073	Unspecified non-aromatic side-chains (C-C and C-N stretching)
IRT-1419; HRB-1419	Unspecified aliphatic side chains (CH_3 deformation) and Glycine (CH_2 deformation)
HRB-929; HCB-956	Unspecified aliphatic side chains (C-C stretching, CH_3 deformation)
CBP-667	Metionine and Cysteine (C-S stretching)
CBT-1031	Phenylalanine (ring stretching)
CBT-1262	Tyrosine (ring stretching)
CBT-1340	Unspecified non aromatic side chain (CH_2 deformation)
HCB-1401	Asparagine, Glutamine (CO_2^- stretching

RBP (Rice Bean Powder without thermal treatment), RBT (Rice Bean Powder with thermal treatment), IRB (protein Isolate from Rice Bean without thermal theatment), IRBT (protein Isolate from Rice Bean with thermal treatment), HRB (Hidrolysate of protein isolate Rice Bean without thermal treatment), HRBT (Hidrolysate of protein isolate Rice Bean with thermal treatment), CBP (Cowpea Bean Powder without thermal treatment), CBT (Cowpea Bean Powder with thermal treatment), ICB (protein Isolate from Cowpea Bean without thermal theatment) and ICBT (protein Isolate from Cowpea Bean with thermal treatment), HCB (Hidrolysate of protein isolate Cowpea Bean without thermal treatment), HCBT (Hidrolysate of protein isolate Cowpea Bean with thermal treatment).

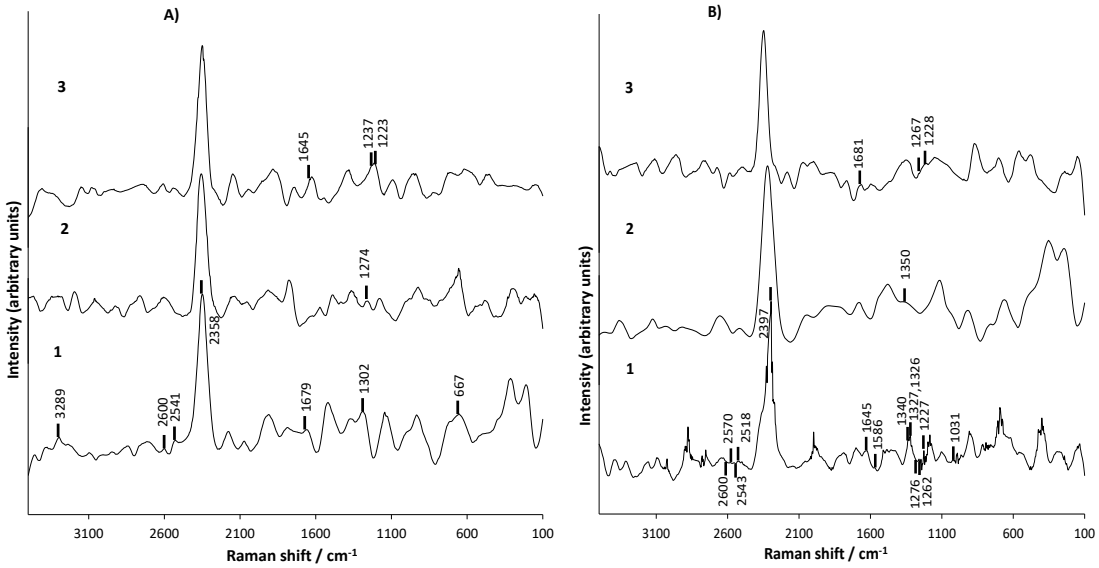

Figure 4. The FT-Raman spectra of the cowpea bean without and with thermal treatment, A) without thermal treatment, B) with thermal treatment. (1) Powder; (2) Isolate; (3) Hydrolysate.

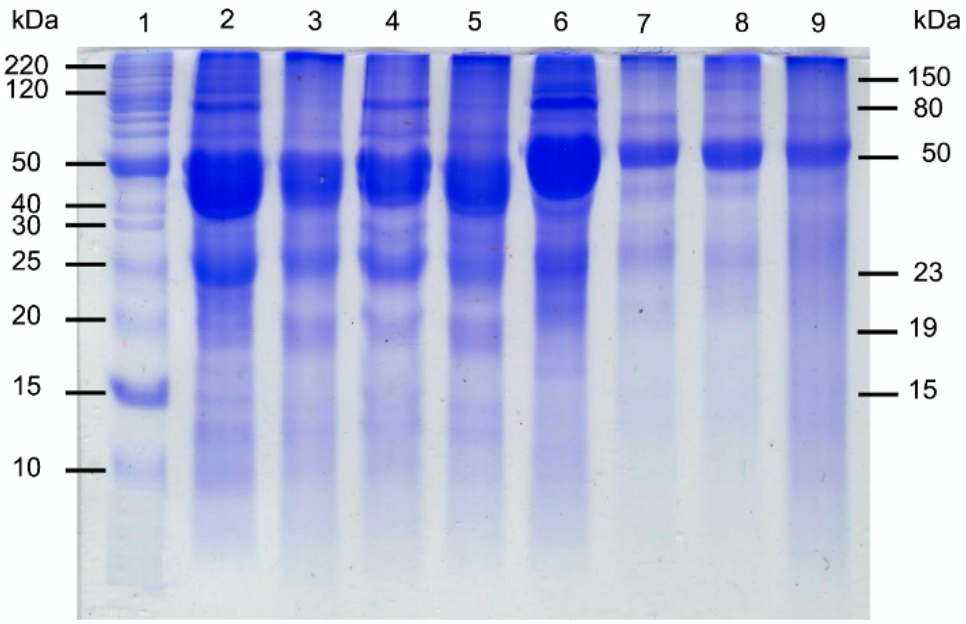

Figure 5. SDS-PAGE electrophoretic profiles of rice and cowpea beans proteins: (1) Molecular weight standard, (2) CBP (Cowpea Bean Powder without thermal treatment), (3) ICB (protein Isolate from Cowpea Bean without thermal theatment), (4) CBT (Cowpea Bean Powder with thermal treatment), (5) ICBT (protein Isolate from Cowpea Bean with thermal treatment), (6) RBP (Rice Bean Powder without thermal treatment), (7) IRB (protein Isolate from Rice Bean without thermal theatment), (8) RBT (Rice Bean Powder with thermal treatment), (9) IRBT (protein Isolate from Rice Bean with thermal treatment).

caused modifications in the secondary and tertiary structure of the proteins, originating that some amino acids residues to be incorporated in more hidden environment due to aggregation and denaturation processes in the protein (Kang *et al*., 2017) which generate modifications in the pattern of the spectra according to the tested sample.

3.6 Polyacrylamide gel electrophoresis (SDS-PAGE)

In the Figure 5 are shown the electrophoretic patterns of the tested samples, in both Vigna species a similar number of bands were observed. However, between both Vigna species slight variations in the molecular weight were observed. For the RBP the molecular weight calculated were, 112 kDa, 93 kDa, 77 kDa, 50 kDa, 30 kDa, 23 kDa and 21 kDa while that in the CBP the molecular weight of the bands were 120 kDa, 101 kDa, 80 kDa, 60 kDa, 50 kDa, 23 kDa and 12 kDa. The variations observed between both Vigna species, apparently are due to changes in the characteristics of the protein in the species and subspecies within the same genus and that could be used as an important marker to distinguish them (Singh *et al*., 2018).

In the electrophoretic pattern of the samples with thermal treatment slight modifications in the number of bands with higher molecular weight were observed (RBT: 93 kDa, 74 kDa, 62 kDa, 50 kDa, 45 kDa, 23 kDa and CBT: 80 kDa, 57 kDa, 50 kDa, 23 kDa, 19 kDa and 13 kDa). In both Vigna species, the band associated to high molecular weight disappeared; according to some authors, a thermal treatment above the 80 ºC generates the dissociation of the subunits and after new covalent disulfide bonds are formed and aggregation process is carried out (Raikos *et al*., 2015).

On the other hand, the isolation process of the protein in both rice and cowpea beans generate a progressive and significant reduction in the number of subunits of high molecular weight, for this reason in addition to the modifications caused by the thermal treatment in the protein isolates a lower number of bands was observed (IRB: 50 kDa, 23 kDa, 45 kDa, 62 kDa; ICB: 58 kDa, 50 kDa, 24 kDa, 23 kDa, and 13 kDa; IRBT: 23 kDa, 50 kDa and 24 kDa and ICBT: 77 kDa, 50 kDa, 23 kDa, 18 kDa and 14 kDa). This reduction in the number of bands have been reported

by other authors, who indicate that the basic and acidic conditions used in the isolation process dissociate several bonds of the protein (Martínez-Palma *et al*., 2015).

In both Vigna species, two bands were maintained after all treatments (23 kDa and 50 kDa), these results indicate that these subunits are stable and according to Singh *et al*., 218, could be part of a common ancestor. In general, modifications in the intensity and distribution of the protein components by effect of the different treatments in the electrophoretic pattern were observed.

Conclusions

Several modifications in the structural characteristics of the proteins of Vigna species were observed using vibrational techniques (FT-IR and FT-Raman spectroscopy), the main observations were, first in both Vigna species protein the β-sheets structure is predominant, however, with the FT-Raman spectroscopy, only in the cowpea bean protein the α-helix structure was observed. Second, the presence of the 3_{10} helix structure in the samples after the hydrothermal treatment was observed. Third, bands associated to several amino acids were observed, a better identification of the bands associated to amino acids in the rice bean protein was carried out. Finally, the FT-IR and FT-Raman spectroscopy are complementary for the identification of modifications in proteins of Vigna species due to the thermal treatment, isolation processes and hydrolysis.

With the SDS-PAGE, in both Vigna species proteins, the same number of bands was observed, however between both Vigna species a slight displacement in the molecular weight of the protein subunits was obtained. Two molecular weight bands are mantained before and after all treatments (23 kDa and 50 kDa), these fractions could be a stable subunits and common ancestor in both species.

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