EFFECT OF MICROBIAL TRANSGLUTAMINASE ON THE PROTEIN FRACTIONS OF RICE, PEA AND THEIR BLENDS

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Running title: Crosslinking of rice flour and pea proteins

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ABSTRACT

BACKGROUND: Transglutaminase (TG) is a transferase that has been used for crosslinking proteins. In general, those interactions are promoted within proteins of the same nature, and very few studies have been conducted for creating new bonds between proteins from different sources catalized by TG. The effect of transglutaminase on the protein fractions of rice flour, pea protein isolate and their blends was studied by using different electrophoretic analysis (simple Sodium Dodecyl Sulphate-PolyAcrylamide Gel Electrophoresis – SDS-PAGE- and multistaking SDS-PAGE under reducing and non-reducing conditions). RESULTS: The transglutaminase induced the disappearance of numerous protein bands as a consequence of the formation of large protein polymers, linked by isopeptidic and disulphide bonds, with reduced solubility. The main protein fractions involved in those interactions were the albumins and globulins, from the pea protein isolate, and the rice flour; and the glutelins were also crosslinked. CONCLUSION: Composite flours containing the rice flour and the pea protein isolate are proposed for obtaining a protein enriched dough with better amino acid balance and also a protein network formed of proteins aggregates of high molecular weight can be created in the presence of transglutaminase.

Key words: rice, proteins, pea, transglutaminase, electrophoresis.

INTRODUCTION

Among the gluten free cereals, rice is the most appropriate for producing fermented products, because of its unique properties.[1-3]. Rice flour has soft taste, colourless, low levels of sodium, easily digestible carbohydrates and low hypoallergenic properties. Nevertheless, despite the described advantages, rice flour shows an important drawback from the technological point of view, since their proteins do not develop the appropriate viscoelastic network necessary to retain the gas produced during the fermentation process, resulting in low quality products.

The addition of transglutaminase (TG) to rice dough has been reported as an alternative to improve the quality of fermented rice based bread by creating a protein network.[4]. The use of this enzyme allowed decreasing the quantity of structuring agents needed to get rice bread with acceptable quality. The transglutaminase (protein-glutamine γ-glutamyltransferase, EC 2.3.2.13) is an enzyme that catalyses the reaction between an ε-amino group on protein-bound lysine residues and a γ-carboxyamide group on protein-bound glutamine residues, leading to the covalent crosslinking of the proteins. This crosslinking may be inter- or intramolecular, yielding an increase in the molecular weight of the protein molecules when intermolecular bonds are formed. [5,6] This reaction has been used for creating crosslinks among proteins from different sources. Proteins such as wheat gluten, soybean proteins, whey proteins, myosin and actomyosin have been reported to be acceptable substrates for the TG, [7-10] modifying the properties of the proteins and thus broadening their applications in foods.

Gluten free products are produced frequently with the addition of different proteins in order to increase their nutritional value. [11-13] Legumes are a good supplement for the cereal based foods, because they increase the protein content complementing the nutritional value of the cereal proteins. Cereals are deficient in lysine, one of the

essential amino acid for the human diet, while legumes show high content of this amino acid. Simultaneously, the cereal proteins are able to complement the legume proteins in the essential amino acid methionine.[14] Although the most used legume protein is the soybean protein due to its valuable functional properties, pea proteins can also be successfully used in bakery products, getting an enrichment in proteins and besides improving the biological value. [15] Moreover, from the technological point of view the addition of proteins from different sources to the rice flour increases the possibilities for the crosslinking by the TG, since the addition of proteins with high content of lysine groups increases the reactivity of the proteins. It has been reported that proteins from different sources interact to wheat proteins in the presence of TG, changing the rheological properties of the wheat flour dough and also their microstructure. [10] The creation of new crosslinks and in consequence the formation of protein polymers might extend the use of the rice flour. It has been reported that the addition of pea protein isolate and TG to the rice flour produces an increase in the storage and loss moduli of the doughs. [16] The protein crosslinking catalysed by TG yielded doughs with more elastic and viscous behaviour. However, changes in the protein fractions of rice flour and pea proteins as affected by crosslinking with TG have not been well studied.

The goal of this study was to determine the effect of the TG on the protein fractions of rice flour and pea proteins by quantifying these fractions, and to understand the nature of the interaction between the proteins in the rice-pea protein blends after the TG treatment using the SDS-PAGE in different conditions.

MATERIALS AND METHODS

The commercial rice flour used was from Harinera Belenguer SA (Valencia, Spain). The rice flour had moisture, protein, lipid and ash contents of 13.4, 7.5, 0.9 and 0.6% (dry basis), respectively. Pea protein isolate was from Trades SA (Barcelona, Spain)

and had moisture, protein, lipid, and ash contents of 6.7, 84.8, 0.9, and 4.5% (dry basis), respectively. Microbial transglutaminase from *Streptomyces* spp. from Ajinomoto Co. Inc. (Tokyo, Japan) (100 units/g) was kindly supplied by Apliena, S.A. (Terrasa, Barcelona, Spain). All reagents in this study were of analytical grade.

Rice dough preparation

Dough was made in a 50g bowl Farinograph (Brabender, Germany). Rice flour (50 g) was mixed with 45 mL of water at controlled temperature (30 °C) for 15 min. Previous studies stated that this mixing time was sufficient for taking place the enzyme reaction. [4, 8-10] In the samples containing pea protein isolate, rice flour was replaced by 5% (w/w, flour-protein blend basis) pea protein isolate. TG, when added, was incorporated at level 1% (w/w, flour-protein blend basis), which corresponded to 1.0 TG unit/g of flour-protein blend. The doughs obtained were used for the determination of the protein content and the rest of the doughs were frozen and freeze-dried.

Protein amount determination

The protein fractions were extracted following a sequential extraction using different solvents, following the method described by Ju et al., [17] with slight modifications. Briefly, albumin-globulin fraction was obtained by suspending 20 g of dough in 100 mL of 5% sodium chloride, then it was homogenized for 5 min and centrifuged at 5,500 x g for 10 min. The procedure was repeated twice for a better extraction and the supernatants were collected. Then, prolamin fraction was extracted by adding 100 mL of 50% 1-propanol to the residue, following the same procedure as in the albumin-globulin extraction. Finally, the glutelins were extracted adding 100 mL of 0.1N NaOH -containing 0.5% sodium dodecyl sulphate (SDS) and 0.6% β-mercaptoethanol (ME) - to the residue.

The protein contents in the supernatants and in the final residue were determined by the micro-Kjeldahl method approved by the AACC. [18] The N:protein conversion factor used was 5.95. [19]

SDS-PAGE protein electrophoresis

Protein extraction

Total proteins were extracted under non-reducing and reducing conditions. The extraction under non reducing conditions was made using the following buffer solution: 0.063M tris(hydroxymethyl)aminomethane (Tris/HCl) pH 6.8, 2% (w/v) SDS, 10% glycerol, and 0.01% (w/v) bromophenol blue. One mL of the buffer was added to the freeze-dried doughs (50 mg of rice or 30 mg in the case of rice-pea protein blend) or to the pea protein isolate (5 mg). Then, the suspensions were vortexed for 2.5 hours and heated in a boiling-water bath for 5 min. After their cooling at room temperature, samples were centrifuged for 5 min at 4,000 x g. The method followed for the extraction under reducing conditions was as described for non-reducing conditions, but buffer solution also contained 3% (v/v) ME as reducing agent. The supernatants containing the proteins were used to perform the electrophoresis.

The protein fractions were extracted by following a sequential extraction with the same solvents used in the determination of proteins amount previously described.

Electrophoresis analysis

Simple SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the supernatant obtained under reducing and non-reducing conditions. Besides, the

supernatants obtained under non-reducing conditions were used for multistacking SDS-PAGE (analytical and preparative).

SDS-polyacrylamide gel electrophoresis was performed in 12% resolving gels with 4% stacking gels according to Laemmli. [20] In multistacking electrophoresis, the acrylamide concentrations of the gels were 4% and 8% (w/v) for the stacking gels and 12% (w/v) for resolving gels.

A Mini Protean II Slab Cell (Bio-Rad Laboratories, Richmond, CA) vertical unit was used. The MW standards were from Bio-Rad (Low range, Bio-Rad Laboratories, Hercules, USA) and consisted of phosphorilase b (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa) and lysozyme (14.4 kDa). The gels were stained with 0.25% Coomassie Brilliant Blue R in methanol/water/acetic acid (4:5:1 v/v) and were de-stained in the same solvent excluding the dying reagent. The gels from preparative multistacking were not stained; instead, they were cut up into pieces and separately submerged into buffer solution containing ME. Then they were vortexed at room temperature for 48 hours and the resulting mixtures were placed into a water bath at 100 ºC for 10 min. Protein composition was analyzed by SDS-PAGE (stacking gel of 4% (w/v) acrylamide and resolving gel of 12% (w/v) acrylamide). Runs were performed in the same equipment as described above. Gels were analysed by an Image Master VDS (Pharmacia Biotech, USA), equipped with an Image Master VDS software (Pharmacia Biotech, USA) providing the integrated optical density (IOD) values.

RESULTS AND DISCUSSION

Protein content in the protein fractions

The sequential extraction of the different protein fractions (albumin, globulin, prolamin and glutelin) and their further quantification provided information about the protein fractions from both the pea protein isolate and the rice flour involved in the crosslinking catalysed by the transglutaminase. The major protein fraction in the rice sample was the glutelin that represents the 77.8% of the total proteins (Table 1), whereas the prolamins were the minor fraction. The albumin-globulin fraction represented the 15.5% of the total protein in the rice flour. The proportion of each fraction agrees with the results reported previously. [17,19]

When the pea protein isolate was present in the rice-protein blend, rice proteins (0.4 g) were replaced by pea proteins (4.2 g), leading to protein enrichment in the blend. The addition of the pea protein isolate produced an increase in the proteins extracted in the albumin-globulin fraction. Conversely, a decrease in the proportion of protein extracted in the glutelin and prolamin fractions was observed. Nevertheless, taking into account that pea proteins have been classified as globulins, [21] the value for the glutelin fraction of the rice-pea blend resulted too high. That result could be attributed to changes in the solubility properties of the proteins, since the extraction process of the protein isolate can affect their properties. [22,23] The proportion of the final residue in the rice-pea protein blend was lower than in the rice sample; thus, the pea proteins were more soluble than the rice proteins in the conditions used.

The activity of the transglutaminase became evident by the change in the protein fractions pattern. It was observed a decrease in the albumin-globulin fraction in both samples (rice flour and the blend rice-pea protein) and in the glutelin fraction extracted from the rice-pea protein blend. In the presence of pea protein isolate the effect of the TG was greater than in its absence, and the more affected fraction was the albumin-globulin. Pea proteins have higher lysine content, what might favour the reaction of the TG because this amino acid is necessary for the crosslinking reaction catalysed by this enzyme. Likewise, the presence of pea proteins might induce either different

218 aggregation of the rice proteins or hinder their aggregation, improving the accessibility of the enzyme to the lysine or glutamine groups involved in the crosslinking.

220 The presence of pea induced greater effect of the TG on all the protein fractions, yielding a reduction of the protein extracted, with a simultaneous increase in the protein

222 content of the final residue. A substantial increase of the protein content in the final residue was observed in both samples after the transglutaminase treatment. This

224 increase was higher in the rice-pea protein blend, where the proportion of protein in the residue increased from 1.6% to 25.3%. Therefore, the crosslinking catalysed by TG

226 lead to the formation of large protein polymers with a reduced solubility of the news polymers formed.

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SDS-PAGE analysis of the total proteins

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Electrophoresis in polyacrylamide gels was performed in order to characterize the

232 protein polymers formed as a consequence of the TG treatment. The electrophoresis was made under different conditions with the purpose of understanding the nature of

234 the interactions among the proteins due to the activity of this enzyme.

236 Under non-reducing conditions, the major bands observed in the rice sample and rice-pea blend were those with MW 14.5-15.7, 22.4-23.5 and 52.8-53.7 kDa corresponding

238 to rice proteins (Figure 1a). In both samples in the presence of transglutaminase, a decrease in the intensity or a disappearance of the protein bands were observed, with

240 the exception of the band of 35.0 kDa, whose intensity increased likely due to the formation of new polymers with different solubility and the band of 15.0 kDa, probably

242 due to peptides that were unable to aggregate due to the transglutaminase activity on different points of the protein chain. The extent of the intensity decrease was greater in

244 the case of rice-pea blends, which agrees with the results previously described for the protein quantification. A decrease in the protein retained on the top of the stacking and

246 resolving gels was also observed in the presence of transglutaminase. The protein polymers unable to enter the gel in the absence of TG increased their MW due to the
248 TG activity, yielding polymers of greater size and lower solubility, which were not extracted and remained in the residue.

250

In order to elucidate if those interactions were due to the crosslinking between the
252 lysine residue and the glutamine residue or were due to other type of interactions like disulphide bonds, the SDS-PAGE was performed under reducing conditions (Figure
254 1b). The analysis of the electrophoresis gels under reducing condition showed lesser amount of streaking but greater number of peaks than non-reducing conditions; which
256 was expected due to the inclusion of ME in the buffer extraction. The rupture of the disulfide bonds between the proteins by the reducing agent yielded shorter protein
258 chains that were able to enter the gel decreasing the amount of protein that was retained in the origin of the stacking and the resolving gel in the absence of
260 transglutaminase (Figure 1b).

The major protein bands in the rice sample appeared at MW 15.1, 22.3 and 32.7 kDa
262 (Figure 1b). These values are close to the values reported by Steenson and Shate in the *Basmati* rice (14.5, 20.4 and 33.1 kDa) and by Villareal and Juliano in *Indica* rice
264 (16, 25 and 38 kDa). [24,25] The major protein bands in the rice-pea protein blend showed about the same MW (Figure 1b), thus probably they came from the rice proteins.

266

In the presence of TG, the gels showed a decrease in the intensity of the majority of
268 the bands. The protein retained on the top of the stacking and resolving gels increased in the presence of TG (Figure 1b).

270 The bands with MW 50.7 and 42.5 from pea protein isolate disappeared after the TG treatment (Figure 1b), which probably corresponded to the acidic polypeptides of the
272 legumin. Larré et al. also observed that after treating pea proteins with TG part of the reaction products did not penetrate the gel, due to the presence of polymers covalently

274 cross-linked. [26] The acidic polypeptides from native legumin are prone to participate in the polymerisation reaction and the basic polypeptides, which have lower MW than the
276 acidic polypeptides, remain almost unchanged during the enzymatic reaction. [27] The acidic polypeptides are hydrophilic and are mainly located at the periphery of the
278 protein, while the basic polypeptides are buried in the centre of the structure. [28]

The crosslinking catalysed by the TG may be intermolecular or intramolecular. The
280 increase in the protein retained on the top of the gels in the presence of TG confirms the intermolecular crosslinking that resulted in an increase in the molecular weight of
282 the polymers. [5,6] The increase in the band intensity on the top of the stacking and resolving gels in the presence of the TG besides the decrease observed under non-
284 reducing conditions indicated that the direct effect of TG was the crosslinking reaction between proteins, and a secondary effect was the formation of disulphide bonds. The
286 formation of disulphide bonds due to the TG activity was also reported by Gujral et al. and Larré et al. [4,29] The crosslinking reaction may bring near the sulphur containing
288 amino acids, making easier the formation of these bonds.

290 **SDS-PAGE analysis of the protein fractions**

292 The effect of the TG on each protein fraction, obtained from a sequential extraction with different solvents, was determined by analysing the electrophoresis pattern of each
294 fraction. The major pea proteins were extracted in the glutelin and in the albumin-globulin fractions (Figure 2, lane 1 and 7), which agrees with the results obtained in the
296 quantification of the protein fractions by Kjeldahl. The prolamin was the minor fraction in all the cases. The pea proteins are classified as albumins and globulins, being the
298 two major globulins named as legumin and thevicilin. [15,30] The high amount of proteins extracted under the conditions used for the glutelins could be ascribed to a change in
300 the solubility of the proteins during the production of the protein isolate, since it has

been reported that the process of the obtention of a protein isolate can modify the properties of the proteins. [22,23]

In the presence of the TG, an evident decrease in the intensity of the bands in the albumin-globulin fraction was observed. In fact, almost all the bands of the rice-pea protein blend disappeared after the TG treatment. At the same time, an increase in the intensity on the top of the stacking and resolving gel was observed (Figure 2, lane 3). This result indicates the formation of protein polymers of higher MW with a concomitant disappearance of the lower MW polypeptides.

The addition of TG did not promoted significant changes in the prolamin fraction. In order to determine the nature of the interaction between the proteins induced by the TG, the glutelin fraction was extracted in two steps, firstly in the absence of ME and in the second step under reducing conditions in the presence of ME. The electrophoretic pattern was the same regardless the extraction conditions. The rice-pea protein blend showed two major bands in the glutelin fraction that appeared at the same MW than those of the rice sample (Figure 2, lane 8 and 9). This band would be the sum of rice and pea protein of the same molecular weight. This may be attributed to the conservatism between the legume and cereal storage proteins, where similar sequences have been found. [31]

In the absence of the reducing agent (fist step), the intensity of the bands showed a pronounced decrease when TG was added, indicating the formation of large polypeptides unable to be extracted in those conditions (Figure 2 lane 9). Conversely, in the presence of the reducing agent, the addition of transglutaminase promoted an increase in the intensity of the bands, thus the rupture of the disulphide bonds favoured the extraction of the large polypeptides that remained in the precipitate. In addition, an increase in the intensity of the band on the top of the stacking and resolving gel was observed in the lanes of the glutelins in the presence of TG (Figure 2 lane 12). Again,

this result confirmed no only the protein crosslinking but also the formation of disulphide bonds. Studies carried out on different proteins described that TG induces a decrease in the intensity or a disappearance of some protein bands and an increase in the protein material unable to enter the stacking or resolving gels, indicative of the intermolecular crosslinking between the proteins. [5,6,32-35]

Multistacking SDS-PAGE

From the analytical multistacking gel was obtained the proportion of proteins (relative IOD) retained in each zone of the gel that depended on their molecular weight (Table 2). The rice-pea protein blend sample showed a higher proportion of proteins with high molecular weight than the rice sample. It might be due to an association of either the pea and rice proteins or the pea proteins among them, obtaining aggregates with higher MW. This result is in agreement with the electrophoretic pattern of glutelin, where some band of rice-pea blend disappeared because they could not be extracted (Figure 2 lane 8). In the presence of the TG it was observed a decrease in the proportion of the protein retained in the 4% gel in both samples, rice and rice-pea protein blend, and in the 8% gel in the case of the rice sample. This decrease could be attributed to the crosslinking reaction that produces polymers of higher molecular weight and more insoluble, unable to be extracted with the solvent used.

The Figure 3 shows the SDS-PAGE pattern of rice-pea proteins eluted from preparative multistacking gels (4, 8 and 12%). The same bands pattern was observed in the three zones of the gels (4, 8 and 12%). This result indicated that all the proteins participated in the formation of aggregates of higher MW. In the presence of the TG, the intensity of all the protein bands decreased and some of the bands even disappeared. The intensity on the top of the stacking and resolving gel in the lane

corresponding to the 4% concentration increased (Figure 3 lane 5). Therefore, almost all the high molecular weight protein aggregates were involved in the crosslinking.

The transglutaminase activity induces the disappearance of numerous protein bands as a consequence of the formation of large protein polymers linked by isopeptidic bonds, but also some new disulphide bonds were formed as a secondary effect of the enzyme activity. The main protein fractions involved in those interactions were the albumins and globulins from the pea protein isolate and rice flour and although in minor extent the glutelins were also crosslinked. Composite flours containing rice flour and pea protein isolate are proposed for obtaining a protein enriched rice dough with better amino acid balance and also a protein network formed of proteins aggregates of high molecular weight can be created in the presence of transglutaminase.

ACKNOWLEDGEMENTS

This work was financially supported by the Spanish Ministerio de Educación y Ciencia Project (MCYT, AGL2005-05192-C04-01) and Consejo Superior de Investigaciones Científicas (CSIC). C. Marco gratefully acknowledges the Ministerio de Educación y Ciencia for her grant.

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Table 1. Study of the effect of the addition of 1% (w/w) TG on rice and rice-pea blend doughs by the quantification of the protein nitrogen content in the different protein fractions (albumin-globulin, prolamin and glutelin).

	Albumin-Globulin (%)	Prolamin (%)	Glutelin (%)	Final residue (%)
Rice	15.5	4.3	77.8	2.4
Rice+TG	10.0	4.9	77.4	7.8
Rice-pea blend	26.8	3.5	68.1	1.6
Rice-pea blend+TG	11.5	3.0	60.2	25.3

480 **Table 2**. Study of the effect of the transglutaminase (TG) on rice and rice-pea blend by the determination of the relative IOD of the protein retained in the different acrylamide
482 concentrations (4, 8, 12%) of the analytical multistacking gel.

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	Relative IOD		
	4%	**8%**	**12%**
Rice	8.10	7.95	83.95
Rice+TG	2.83	2.27	94.90
Rice-pea blend	12.88	10.52	76.60
Rice-pea blend +TG	8.67	11.26	80.06

486

FIGURE CAPTIONS

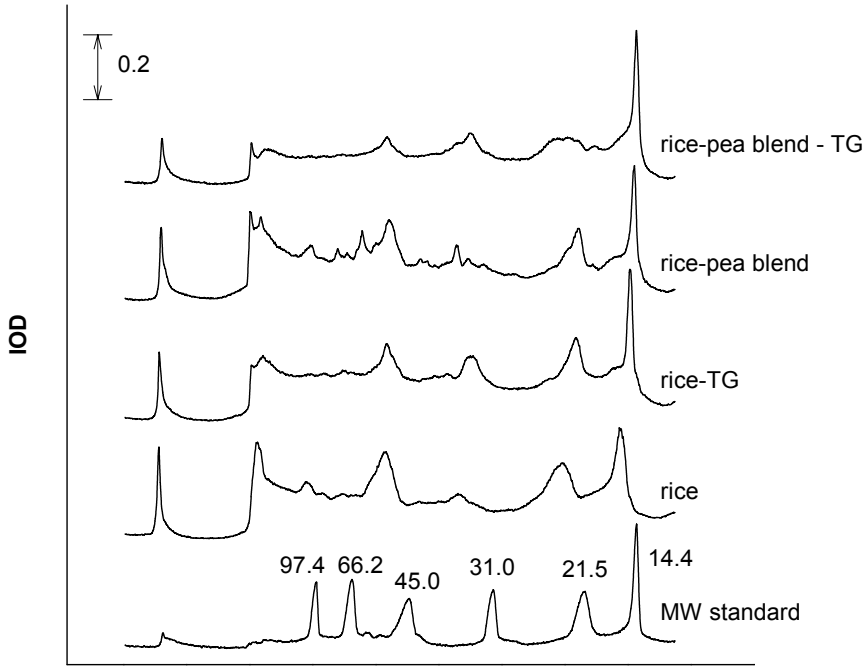
Figure 1. Electrophoregrams obtained from the analysis of the SDS-polyacrylamide gels of the proteins from rice and rice-pea blends in the absence and the presence of 1% (w/w) transglutaminase. The values in the MW standard are expressed in kDa. (a) unreduced conditions; (b) reduced conditions.

Figure 2. SDS-PAGE analysis of the protein fractions in pea protein isolate (without TG) (lanes 1, 4, 7, 10) and in rice-pea blend without (lanes 2, 5, 8, 11) and with TG (lanes 3, 6, 9, 12). Albumins-globulins (lanes 1, 2, 3), prolamins (lanes 4, 5, 6), glutelins step 1 (lanes 7, 8, 9), glutelins step 2 (lanes 10, 11, 12). MW standard (lane 13).

Figure 3. SDS-PAGE analysis of rice-pea blend from preparative multistacking gels. Concentrations of 4, 8 and 12% without TG (lanes 2, 3 and 4, respectively) and with TG (lanes 5, 6 and 7). MW standard (lane 1).

502 Figure 1. Marco et al.

504 1 (a).



506 1 (b).

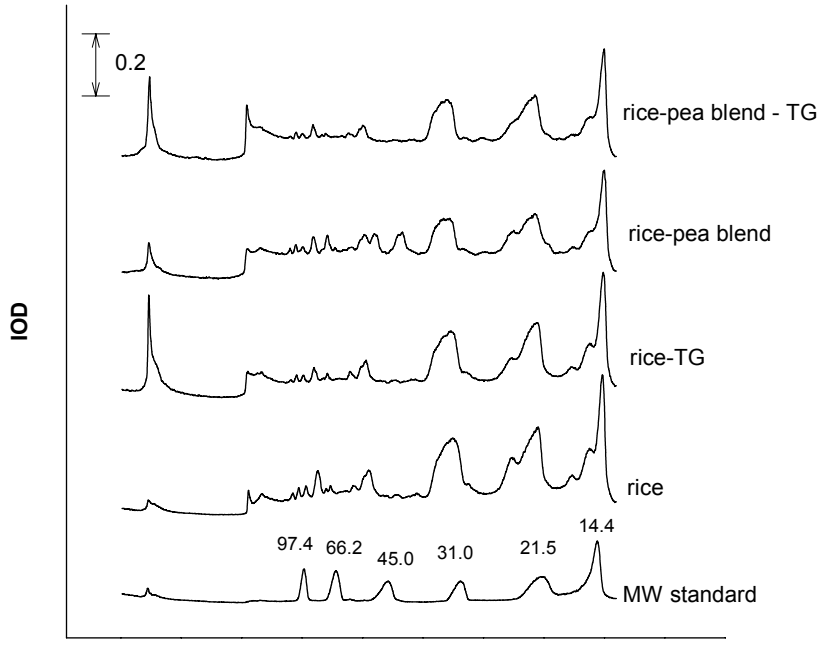


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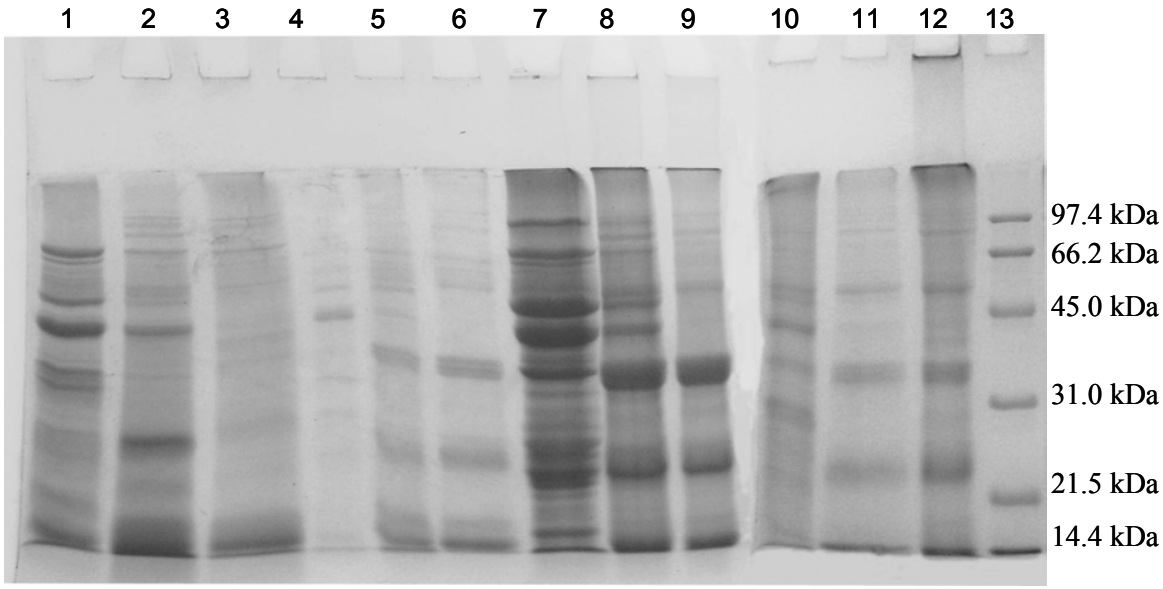
Figure 2. Marco et al.



534 Figure 3. Marco et al.

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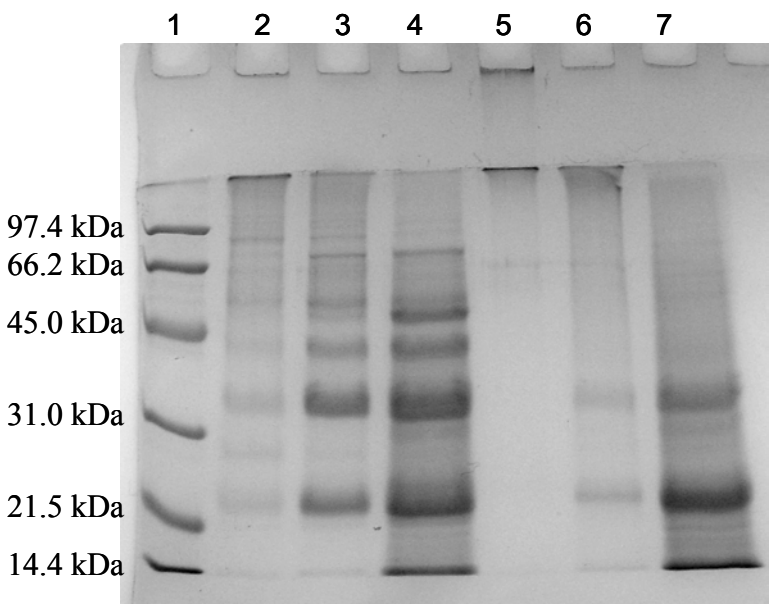

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