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Development of a Competitive Enzyme Immunoassay Technique for the Detection of Soy Traces in Meat Products

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Abstract: The aim of this work was to develop a competitive enzyme immunoassay technique, to detect the presence of traces of soy in meat products. Specific rabbit polyclonal antiserum against soy protein was used as primary antibody. The optimal antigen concentration to be immobilized on the plate and the concentration of primary antibody to be used in competition was determined. The calibration curve was fitted using increasing concentrations of an extract of soy product. The soy product was extracted with Tris-HCl buffer 0.0625M with 3% sodium dodecylsulfate and 2% mercaptoethanol. The working range used in the enzyme immunoassay to detect soy was 9-280ppm SP with adequate linearity (R^2: 0.9880). All validation parameters studied were appropriate. Commercial samples of meat products were analyzed with this enzyme immunoassays and a commercial ELISA kit. Significant differences were observed in the quantitative results obtained with both methods; nevertheless the developed enzyme immunoassay could be used as screening method.

Keywords: ELISA, Allergens, Soy Detection, Meat Products

1. Introduction

In the manufacture of meat products often extrinsic proteins as bovine or porcine plasma, soy products, different dairy products (caseinate, whey, skim milk powder, etc.), collagen, gelatin, are added [1]. These proteins are used as water retention agents and improve the emulsification of fats. They are good coagulants during cooking and improve shine and moisture of the product. Some of the proteins previously mentioned are food allergens and therefore constitute a risk for allergic patients, mainly when these proteins are not declared as ingredients in the food labels. López L et al, 2010 have detected protein ingredients that were not declared in meat products[2].

There are eight food groups (The Big-8) that are responsible for 90% of food allergies: milk, egg, soy, wheat, peanuts, tree nuts, fish and shellfish [3].

The presence of undeclared allergenic proteins in meat products may be due to different reasons. They may have been added as ingredients or additives but are not declared because they are not approved in these products. When the ingredients or additives are approved, a voluntary omission may occur in the declaration, e.g.: the formulation is modified but non-updated labels are used. The omission may be involuntary, e.g.: when the ingredients or additives composition is unknown. Another reason may be due to cross-contact, e.g.: when product lines are shared for processing different kind of products.

There is a need of methodology that enables the detection of extrinsic allergenic proteins in meat products. The most common methodology for the analysis of food allergens is ELISA. In Argentina commercial kits are available from different companies. However, the cost of these kits is rather high.

Therefore the aim of this study was to develop a competitive enzyme immunoassay, to detect the presence of traces of soy in meat products.

2. Materials and Methods

2.1. Samples Analyzed

2.1.1. Soy Product

The soy product contains 63% protein. The percentage of proteins was obtained by Kjeldhal method.

2.1.2. Model Systems of Raw Meat

Two model systems of raw meat with the addition of soy product were prepared in the laboratory. The model systems were: 20 and 75 ppm of soy proteins (SP) in mixture with raw meat.

2.1.3. Commercial Meat Product Samples

- 1: Hamburger. Ingredients: Bovine meat.
- 2: Hamburger. Ingredients: Bovine meat.
- 3: Hamburger. Ingredients: Bovine meat.
- 4: Sausage. The list of ingredients was not available.
- 5: Cooked pork product. The list of ingredients was not available.

Samples 1, 2 and 3 are commercial hamburgers made with raw ground beef. They were not heat treated. Samples 4 and 5 are meat emulsions that were heat treated (this treatment was a pasteurization).

2.2. Protein Extraction from Soy Product and Model Systems of Raw Meat

Thirty mg of soy product and 300 mg of minced meat were weighed for the protein extraction.

Two mL of total protein extractive solution was added. This solution contains 0.0625M Tris-HCl with 3% sodium dodecylsulfate (SDS) and 2% mercaptoethanol (ME). The tubes were heated in a water bath at 100°C for 5 minutes. After 2 minutes in the bath it was shaken with a rod. The contents of the extraction tubes were transferred to plastic tubes and centrifuged at 3000rpm for 15 minutes (CAVOUR 3216-D centrifuge, Argentina). The supernatants were stored at -20°C until analysis.

The final concentration of proteins in each extract was approximately10-30 mg/mL of proteins.

The method of Lowry [4] was used for the quantification of SP in the extract and for the calculation of the recovery. The proteins were extracted as previously described; 2 mL of acetone was added to 0.2 mL of the extract to precipitate the proteins. It was constantly shaking with a Vortex (Virtis "23", New York) and then was centrifuged at 3500 rpm for 25 minutes. The precipitated proteins were resuspended and washed twice with acetone. It was reconstituted with 0.5 mL 1N NaOH with shaking 30 minutes at 37°C, finally the protein was dissolved with 2 mL of distilled water. To perform the Lowry method 0.2 mL of this solution were taken. Bovine serum albumin was used as the protein standard for the calibration curve [5].

The percentage of recovery of SP in the extract was calculated from the real concentration.

2.3. Optimization of the Competitive Enzime Immunoassay

The optimal concentration of antigen (soy) to be immobilized on the plate and the optimal dilution of primary antibody (rabbit polyclonal antiserum specific for SP) to be used in competition was determined. Polyclonal antiserum obtained in rabbits immunized with soy and obtained according to [6], was used.

2.4. Plate Coating

Microplate plates (Maxisorp ®, NUNC, Denmark) were used. For this, 100 µL per well of two different concentrations of antigen were seeded. The concentrations were: 1 µg of SP / 100 µL or 10 µg of SP / 100 µL of Carbonate / Bicarbonate buffer, pH: 9, 6 (Buffer Sodium Carbonate 0.015M, 0.035M sodium bicarbonate, pH: 9.6). The plate was then incubated in a humid chamber, in the dark at 4°C for 24 h. The plate was washed 5 times with wash solution (0.9% w/v NaCl and 0.0125% v/v Tween 20 in water). Two hundred µl of blocking solution (1% w/v bovine gelatin and 0.1% v/v Tween 20 in TBS) were seeded into each well. It was incubated for one hour in a humid chamber, in the dark at 37°C, with shaking. The plate was washed 5 times with wash solution. Subsequently, 100 µL of different dilutions of the primary antibody diluted with TBS buffer with 0.1% v/v Tween 20 and 3% polyethylene glycol were seeded. Primary antibody dilutions that were assayed were between 1/156 and 1/10000. In the wells corresponding to the blank (blank 1 and blank 10) only the buffer used for the dilution of the primary antibody was seeded. It was incubated for one hour in a humid chamber, in the dark at 37°C with shaking. The plate was washed 5 times with wash solution. One hundred µl of Bio-Rad alkaline phosphatase conjugated Anti-IgG secondary antibody (obtained in goats immunized with purified rabbit IgG) was seeded in the wells. The secondary antibody was diluted 1:3000 with TBS buffer with 0.1% v/v Tween 20 and 3% polyethylene glycol. It was incubated for one hour in a humid chamber, in the dark at 37°C with shaking. The plate was washed 5 times with wash solution. Finally, 100 µl of a solution containing 1 mg/mL paranitrophenyl phosphate in a buffer containing 10% v/v diethanolamine and 0.01% magnesium chloride, pH: 9.8 were seeded. It was incubated 20 minutes in a humid chamber, in the dark at 37°C with shaking. Absorbance was measured on an ELISA microplate reader (ELISA RT-2100C, Rayto, China) at 405nm. The absorbance values were corrected with the average absorbance corresponding to the blank. Corrected absorbance versus ln 1 / dilution of primary antibody curves were plotted, using a Microsoft Excel 2010 spreadsheet.

2.5. Validation of the Competitive Enzyme Immunoassay for the Detection / Quantification of Soy Traces in Meat Products

2.5.1. Linearity

For the determination of the linearity of the method, increasing concentrations of a soy product extract with 0.0625M Tris-HCl buffer with 3% SDS and 2% of ME were used. The curve had five points 0; 0.01; 0.03; 0.1 and 0.3µg

SP / mL Carbonate / Bicarbonate buffer, pH: 9.6. For each point of the curve a dilution of the original extract was performed but the SDS and ME concentration remained constant. In this way the components of the extractive solution were diluted 1: 175 at all points of the curve. Dilutions were performed in carbonate / bicarbonate buffer, pH: 9.6.

Seventy five µL of the dilution of the primary antibody selected in the test optimization, and 75µL of each of the dilutions of the previously prepared curve points, were pre-incubated. In addition, two controls were prepared; a "non-specific control" (NS) containing 200 µL of the buffer used to dilute the primary antibody, and a "maximal binding" (M) control containing: 100 µL of the buffer used to dilute the primary antibody and 100 µL of the primary antibody selected in the test optimization. The preincubates were incubated at 4°C in a humid chamber and in darkness for 24 h. Also, an ELISA plate was sensitized by sticking the concentration of antigen (soy) that was previously selected in the test optimization. It was then incubated in a humid chamber, in the dark at 4°C for 24 h. The plate was washed 5 times with wash solution. Two hundred µl of blocking solution were seeded into each well and incubated for one hour in a humid chamber, in the dark at 37°C, with shaking. The plate was washed 5 times with wash solution. Subsequently, 100 µL of the preincubates were seeded. It was incubated for one hour in a humid chamber, in the dark at 37°C with shaking. The plate was washed 5 times with wash solution. The protocol was followed as previously described in the plate coating item. The absorbance values were corrected with the mean absorbance corresponding to non-specific control NS. An absorbance calibration curve was constructed, corrected absorbance versus ln µg of SP / mL.

The tests used for the statistical analysis of the results were: Barlett method, for homogeneity of variances and linear regression analysis [7].

2.5.2. Limit of Detection and Limit of Quantification

To determine the limits of detection and quantification of the method, a sample of minced meat without analyte (soy) was used. It was extracted five times as described above. Each extract was analyzed in duplicate, as described above, by pre-incubating the dilutions 1: 175 with carbonate/bicarbonate buffer, pH: 9.6. The analyte concentration in each sample was calculated according to the formula (1). The mean value of the analyte for the minced meat sample without analyte and the corresponding standard deviation were calculated. The detection limit was calculated as the mean value plus three times the standard deviation. The quantification limit was calculated as the mean value plus ten times the standard deviation.

The amount of SP in µg / g of meat product is calculated according to the following formula:

(1) Amount of SP in the meat product-µg/g=$\frac{\text{amount of soy prot− µg (1) x V−µL (2) l x 1000−mg (3)}}{\text{5,7−µL (4) x P−mg (5)}}$

(2) µg of SP interpolated in the calibration curve.

(3) Volume of supernatant obtained when extracting the meat product with extractive solution of total proteins: 1600 µL

(4) 1000 mg: to express the content in 1000 mg of meat product.

(5) 5.7 µL. It is the volume of extract that is taken from the 1600 µl of supernatant and diluted 1: 175. 5.7 µL are brought to 1000 µL with Buffer Carbonate / Bicarbonate; PH 9.6.

(6) P: 300 mg. It is the weight of meat product that is extracted with extractive solution of total proteins.

2.5.3. Precision

To evaluate the intraday precision of the method, three samples of meat product containing equal amount of analyte (150 ppm of SP) were analyzed. Each sample was extracted as described above (n=3). Each extract was analyzed with the competitive enzyme immunoassay in duplicate as described above, by performing the 1: 175 dilution of each of the samples prior to the preparation of the preincubates. The analyte concentration in each sample analyzed was determined according to the formula (1).

For the statistical processing, the analyte values of the three samples were averaged, standard deviation and coefficient of variation (CV) were calculated. This CV corresponds to the precision of the method in the day.

In order to evaluate interdays precision, the same procedure as previously described in the intraday test was performed on three different days (n=9). For statistical processing, the mean, standard deviation and coefficient of variation (CV) of the nine values obtained were calculated. The CV corresponds to the precision of the method between days.

It was adopted as an acceptance criterion that the CV of the intraday precision and the CV of interdays precision did not exceed 15% [8].

2.5.4. Recovery

Two model systems of minced meat mixed with 75 and 20ppm of SP were analyzed to evaluate the recovery of the method.. They were extracted in triplicate as described above. They were analyzed in duplicate as described above, by performing the 1: 175 dilution of each of the samples prior to the preparation of the preincubates. The analyte concentration in each sample analyzed was determined according to the formula (1). For each model system the three analyte values were averaged.

The percent recovery was calculated by the formula described below (2).

(2) % Recovery = obtained value x 100 /real value

- Obtained value: concentration of SP obtained when applying the enzyme immunoassay for SM of 75 and 20ppm of SP

-Real value: 75 or 20ppm of SP.

The recoveries of the two model systems were then averaged. Recovery values between 70-130% were considerate adequate values [9].

2.6. Commercial ELISA Kit

The commercial meat products were analyzed with the competitive enzime immunoassay and also with the ELISA Ridascreen® Fast Soya from R-Biopharm. All samples were assayed in duplicate following the protocols of this kit.

The detection limit (DL) and quantification limit (QL) for this kit were: DL: 0.31 ppm SP and QL: 2.5 ppm SP with a quantification range of 2.5-20.0 ppm SP

3. Results and Discussion

3.1. Quantification of SP in the Extract

The concentration of SP obtained in the extract of total proteins was 8.9 mg of SP / mL of extractive solution. The real concentration in that extract was 9.4 mg of SP / mL of extractive solution. The recovery rate was 95%, it means that 95% of the SP were soluble in the extractive solution of total proteins.

3.2. Optimization of the Competitive Enzyme Immunoassay

Figure 1 shows the two curves obtained for the determination of the optimum concentration of soy antigen and the primary antibody dilution to be used in the final assay.

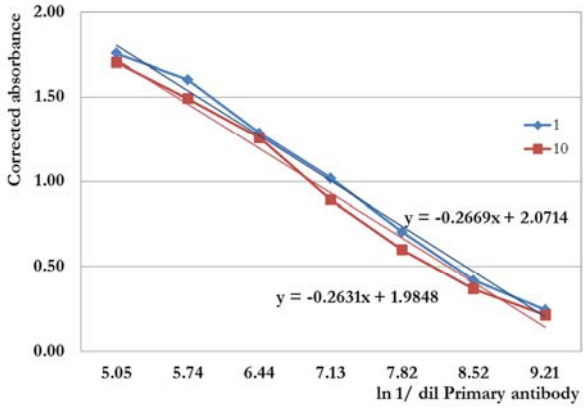

Figure 1. *Curves obtained for the determination of the optimal concentration of soy antigen and the dilution of primary antibody to be used in the final competitive enzyme immunoassay.*

Figure 1 shows the curves corresponding to 1 µg of SP / 100 µl of Carbonate / Bicarbonate buffer, pH: 9.6 and 10 µg of SP / 100 µl of Carbonate / Bicarbonate, pH: 9.6 which were obtained in the test optimization.

The curve with the highest slope was chosen for the selection of the optimal concentration of antigen to be used in the test. (1µg of SP / 100µL of buffer)

In order to obtain a method with adequate sensitivity, the optimal dilution of primary antibody to be used in the competition was chosen in the area most sensitive to changes in the curve of 1µg of SP / 100µL of buffer. The dilution of primary antibody selected for use in the competition was 1/1250. (Ln 1 / primary antibody dilution: 7.13).

3.3. Validation

3.3.1. Linearity

SDS and ME have been globally recognized for their effective extraction [10]. They are useful for the solubilization of insoluble proteins. The ME cleaves disulfide bonds formed between the cysteine residues of proteins and SDS facilitates the solubilization of proteins by altering non-covalent bonds [11].

ME and SDS interfere with the antigen-antibody reaction of the test, for this reason the dilution of the extractive solution which did not affect the antigen-antibody binding was evaluated. Some researchers have observed that generally concentrations of this buffer sufficiently dilute do not influence the performance of the test [12].

Three dilutions of extractive solution in carbonate / bicarbonate buffer, pH: 9.6 (1:50, 1: 100, and 1: 175) were tested by performing the competitive enzyme immunoassay as previously described. Absorbance values at the 1:175 dilution were found to be similar to those obtained in the "maximal binding" (M) wells. In contrast with the dilutions of 1:50 and 1: 100 the absorbance values were lower than the values corresponding to M. This involves quantification of analyte in a solution that does not contain it (competitive assay). The lower values of absorbance are due to an interference of extractive solution components in the antigen-antibody binding and not to the presence of analyte.

According to these results for each point of the curve, a dilution of the original soy extract was performed, but the concentration of SDS and ME was kept constant. These concentrations correspond to a 1: 175 dilution of the extractive solution.

Five points were used to establish linearity 0; 0.01; 0.03; 0.1 and 0.3 µg SP / mL (Figure 2).

A test of homogeneity of variances was applied to the values of corrected absorbances obtained for each level of concentration and no significant differences were found between the variances of the different levels analyzed.

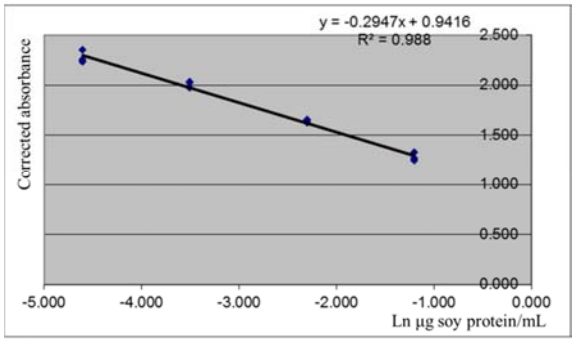

Figure 2. *Calibration curve: Absorbance corrected vs. ln µg of SP/mL.*

The linearity test was performed using the professional Infostat version 2004d.1 developed by the National University of Córdoba. A value of F = 1.84 (CM linearity deviation / CM pure error) and p = 0.2195 was obtained. It was concluded that the range 0.01; 0.03; 0.1 and 0.3 µg SP / mL showed lineal. The obtained line had a slope of -0.29

with a lower limit 95% (LL) of -0.32 and an upper limit 95% (UL) of -0.27, intercept 0.94 with LL: 0.87 and UL: 1.01 and a correlation coefficient of 0.988. The formula (1) presented above was applied to the limit values of the calibration curve (0.01 µg of SP / mL and 0.3 µg of SP / mL), in order to calculate the working range for the SP in meat products. The working range was 15-420 ppm of SP in meat products.

3.3.2. Detection and Quantification Limits

The values of the detection and quantification limits were 9.0 and 18.0 ppm of SP, respectively.

3.3.3. Precision

The intraday and interdays precision of the method expressed as coefficient of variation (CV) were 7.8 (n = 3) and 12.7 (n = 9), respectively. These precision values are adequate.

3.3.4. Recovery

Model systems of raw meat with 75 and 20 ppm SP were analyzed. Results were lower than expected (23 and 17 ppm, respectively). This indicates that recovery from this assay is not adequate because of the interference of the meat matrix either in the extraction of the SP and / or in the detection of such proteins.

According to the study of Cellerino et al., [13], some commercial ELISAs also present values much lower than expected in model systems of raw meat analyzed. Results obtained using R-Biopharm and Veratox-Neogen kits were lower than real values. For example the result obtained in the model system with 250 ppm of SP concentrate (157. 5 ppm of SP) using Veratox® Quantitative Soy Allergen Test from Neogen, was 21.6 ppm of SP. Also in a model system of cooked boneless ham with 250 ppm of SP concentrate the result was 13,4 ppm of SP using the same kit.

The ELISA method is an important tool to detect allergens in food, but it must be taken into account that different food matrices can affect the recovery of the method, and it is not possible to recognize, in the majority of cases, the compound of the food that interferes with the test [14].

3.4. Analysis of Commercial Meat Product Samples

Table 1 presents the results obtained in the determination of SP in commercial meat products, using the R-Biopharm kit and the competitive enzyme immunoassay developed.

Table 1. *Results obtained in the determination of SP in commercial meat products using the R-Biopharm kit and the competitive enzyme immunoassay developed.*

Samples	Ridascreen® Fast Soya from R-Biopharm (ppm, SP)	Competitive Enzime inmunoassay developed (ppm, SP)
1	>20,0	39,1±3,1
2	5,2±1,6	29,0±3,5
3	<2,5	<18,0
4	>20,0	>280,0
5	>20,0	>280,0

It has been observed in different studies that the results between different commercial ELISAs may be different. Although Elisa method is accepted as standard method for allergen measurement, these results seemed to vary from manufacturer to manufacturer. This variation could be due to the lack of standardization of the method, the calibration material used, the extraction solutions used or the antibody specificity [15]. Therefore the quantitative results between these two methods cannot be compared.

In sample 1 the result obtained with R-Biopharm exceeds the upper limit of the calibration curve whereas in the competitive enzyme immunoassay the result obtained is within the working range of this method. In sample 2, quantifiable results were obtained within the working range of each method. In sample 3 both methods had results below the quantification limit. In samples 4 and 5 the results obtained are greater than the upper limit of the calibration curve of each method. In a previous work all the samples were analyzed using SDS-PAGE. SP were not detected in samples 1, 2 and 3 but they were detected in samples 4 and 5 (results not published). The limit of detection of SP in meat products using SDS-PAGE is 5000 ppm soy isolated [1]. It means that the samples 4 and 5 contain soy as an ingredient.

The sensibility of the competitive enzyme immnunoassay developed is higher than the sensibility of the SDS-PAGE.

If a sample shows a positive result with the competitive enzyme immunoassay, it is not necessary to use a commercial ELISA kit because the sample contains soy. The cost of the competitive enzyme immunoassay developed was calculated in December 2016. It was 0.60 U.S dollars per well. At that time a commercial kit had a market value of 13.00 U.S. dollars per well. So the enzyme immunoassay developed has a considerably lower cost than commercial kits. Therefore, this could be used as screening method, to analyze samples in which a possible cross-contact with soy is suspected. If negative results are obtained with this methodology, it should be confirmed with a commercial ELISA kit of adequate sensitivity, to ensure the absence of SP.

4. Conclusion

This competitive enzyme immunoassay could be used as screening method. If in a sample the result is positive with these competitive enzyme immunoassays, the presence of soy can be confirmed. However, if the result obtained is negative (less than the limit of quantification of these methods) it is necessary to confirm the result with a commercial ELISA soy kit of adequate sensitivity.

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