Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2015, 7(1):926-930

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Extraction and characterization of certain food allergen proteins of animal origin

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ABSTRACT

Because they are widely used for human consumption, cow's milk and hen eggs allergy are of the most commonly implicated causes of food allergic reactions. To characterize allergen proteins of these two products, ovalbumin, conalbumin, lysozyme, ovomucoïde, serum-albumin, β-lactoglobulin, whole caseins, αs1-casein, β-casein and k-casein are extracted, isoelectric points, temperatures denaturation, carbohydrate and protein rates are determined, relationships between different physicochemical parameters are defined, amino acids quantity of protein hydrolysates are demonstrated and relative molecular masses are evaluated. Results reveal the existence of thermoresistant and thermolabile allergen proteins, most of them have an acid isoelectric point and carbohydrate portion and rich in cystein and tyrosin, what assure to the protein a geometric compact structure resistant to the unfolding provoked by heat treatments. All of them are of low relative molecular weight. There is a relationship between carbohydrate rate and temperature denaturation and it is confirmed that glycan fraction protects glycoproteins from thermal denaturation. Trophallergens characterized by an acid isoelectric point, low relative molecular weight, high content of carbohydrate and rich of cystein and tyrosin residues are necessary thermostable. When trophallergens have a basic or near of neutrality isoelectric point, an unimportant content of glycan portion and when they are of low relative molecular weight and poor in cystein and tyrosin, they are necessary thermolabile.

Keywords: Food allergy; Allergen proteins; Extraction; Characterization; Thermostable; Thermolabile.

INTRODUCTION

Proteins are an important component in human nutrition, they enter in composition of biological substances, foods are typically derived from animal and vegetable sources, we interest to these components because of the nutritional value, their effect on human body and the toxicity that can be engendered. Ingestion of certain protidic substances by genetically predisposed person to not tolerate the consumed product cause hypersensibility reactions or food allergy. The prevalence of allergic disorder s has been increasing in the past 10-15 years [1]. It is has emerged for many raisons especially globalization of commercial transaction[2] changes of eating habits and evolution of agro-food technology, that's why allergy became a serious public health issue [3]. Allergen proteins are called trophallergens and they are responsible of different clinical manifestations including cutaneous, respiratory and gastrointestinal reactions, while anaphylactic shock seems to be the severest manifestation [4]. These molecules are generally of low molecular weight glycoproteins with an acid isoelectric point. In addition, they tend to be resistant to usual food processing and preparation conditions. These proteins are comparatively resistant to heat and acid treatments, proteolysis and digestion and they have the capacity to link a specific IgE [5]. Other physicochemical properties are not well known. reduction or elimination of proteins allergenicity can be possible through biotechnology and industrial processes. Many studies have been conducted to develop hypoallergenic foods. In that context this work is consecrated to the characterization of certain food allergen proteins of animal

origin.

EXPERIMENTAL SECTION

The cow and hens which are sources of these two products are of known Algerian species. Fresh cow's milk and hen eggs were purchased from a local store, as soon as possible milk is taken it is cooled to 4°C and stored at this temperature for up to 1 hour. Before, the fat was removed by centrifugation for 20 min at 1500g/15°C. For eggs, blended native egg white was filtered through three layers of cheesecloth to remove pieces and chalazae, prepared samples were stored at -20°C. Prior to analysis, frozen samples were thawed overnight in a refrigerator maintained at 4°C.

1- Allergen proteins extraction

Cow's milk allergen proteins extraction

The whole casein was prepared from skim-milk by slow acidification with 0.1N HCl to pH 4.6 AT 25°C [6]. The αs1-casein, β-casein and k-casein fractionation and β-lactoglobuline isolation from whey proteins are obtained by Simon Roe described methods [7]. Serumalbumine is prepared by salt fractionation method of Aschaffenburg and Dreway[8].

Hen egg allergen proteins extraction

Ovalbumin, ovomucoid, conalbumin and lysozym are obtained by ammonuim sulfate fractionation of native egg white [9].

2- Physicochemical parameters determination

Protein rates were determined using Bradford method. Determination of carbohydrate rates was carried out by the anthrone method [10]. For the isoelectric points, the acetic acid 0,1M or NaOH 0.1M was added to protein allergen solutions progressively and attentively until precipitation of proteins. The values were indicated on pH-meter. Concerning the temperature denaturations of protein allergens, the test tubes containing protein allergen solutions were introduced in a water bath adjusted at 30°C.The temperature was increased progressively and attentively, 1°C each 3mn, until precipitation. The results were expressed by the different repetitions average. The statistical analysis was achieved by mean of Minitab 16.

3- Amino acids composition of allergen proteins

Amino acids composition of proteins was determined in SGS MULTILAB after hydrolysis with 6N HCl at 110c for 18 hours accoroding to the method of Ozols [11]. Using Reversed-Phase High Performance Liquid Chromatography (HPLC) with pre-column derivatization, under the following conditions; Pre-colonne Atlantis dC 100*4.6mm, porosity 3um, column température : 30°C, debit : 0.2ml/min.

4- Allergen proteins Molecular weight determination by SDS-PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (12%T) was carried out using the discontinuous buffer system under denaturing conditions (with 2-mercaptoethanol) described by Laemmli [12] at a constant current of 200 V and 25 mA. An appropriate volume of the protein sample was mixed with an equal volume of sample buffer (0.0625 M TrisHCl, pH 6.8, 2 % SDS, 10 % glycerol, 0.002 % bromophenol blue, with 5 % β-mercaptoethanol) and submitted to heat treatment for 5 min in a boiling water bath prior to be applied to the gel. Samples allowed to cool to room temperature, finally centrifuged at 10000 g for 5 min to remove any insoluble materials causing streaking during electrophoresis. After gel polymerization, 30 µg protein were applied to each lane in the gel. After electrophoresis gels were stained for 30 min using 0.1% Coomassieblue R-250 (Bio-Rad) and then distained using a distaining solution of glacial acetic acid, methanol and water (1:4:9).

Molecular weights determination was carried out by using standard protein marker, molecular weight range; KDa, sigma, according to the method described by Weber and Osborn [13].

RESULTS AND DISCUSSION

Table 1 and 2 summary the comparison of physicochemical parameters of cow's milk and hen eggs allergen proteins. ``P`` values calculated by the analysis of the variance in a criterion of fixed model classification are null. There are very highly significant differences between each of these physicochemical parameters. Isoelectric points, temperature of denaturation, carbohydrate rate and proteins rate are successively; 4.11 to 10.94, 52.6°C to 98.88°C, 1.39% to 21.66% and 65% to 94.83%. Concerning temperature denaturation, results are in agreement with those reported by Monret-Vautrin [14] et Cayot et Lorient [15] and for isoelectric points, results are agree with those

reported with Hefle [16]. It is shown that all the isoelectric points are acidic except lysozyme who have a basic isoelectric point. All allergen proteins have a glycan moiety. Ovomucoid is the most thermoresistant, cow's milk whey proteins are denaturalized at temperatures superiors than 76°C. During their extraction, caseins are precipitated with chlorhydric acid, acidification cause modification in micelle structure, caseins became instable, and it is shown that low temperatures can alterate caseins known to be more thermoresistant when they are in their natural environment. Conalbumin and κ-casein are the poorest in proteins amount, ovalbmin and β-lactoglobulin are the richest.

The relationships between the different physicochemical properties of cow's milk and hen eggs allergen proteins are shown in table 3. The correlation coefficients, coefficients ofdetermination and linear regression equations were illustrated. The correlation coefficients between the temperature denaturation and the carbohydrate rate is highly significant r= +0.84 ($p \leq 0.01$). When the carbohydrate rate is considerable, the temperature denaturation is high, it is revealed that glycan moiety increase the thermal resistance, it contributes to the stability of the allergen proteins and the protection of glycoproteins against heat and this result is in accord this of Pelmont [17].

Table 4 indicates the amount of amino acids in gramme per gramme of cow's milk and hen eggs protidic extract, determinated by Reversed-Phase High Performance Liquid Chromatography (HPLC),tryptophane is destroyed by acidic hydrolysis. In this analysis we are interested to cystein and tyrosine s quantity, abundance in these two amino acids provokes creation of disulfide bonds, hydrogen bonds and hydrophobic interactions, tyrosin is an hydrophobic and aromatic amino acid, responsible of hydrogen bonds creation with other residues, those links are breakable and unstable but this fragility is composed by their high number which insure an essential role in the maintenance and the stabilization of the three-dimensional structure of the protein. Disulfude bonds are formed by cystein-cystein condensation, they characterize tertiary structure of proteins, they are not destroyed by high temperatures during denaturation processes, but in the case of caseins, it is important to indicate that thialate form increasing during heat treatments is responsible of disulfude bonds breaking by nucleophilic attack.

According to carbohydrate rate, temperature denaturation and cystein and tyrosin amount of ovomucoid, b-lactoglobulin and serum albumin, it is specified that a protide which have an increasing amount of carbohydrates and rich in cystein and tyrosin have necessary a considerable temperature denaturation.

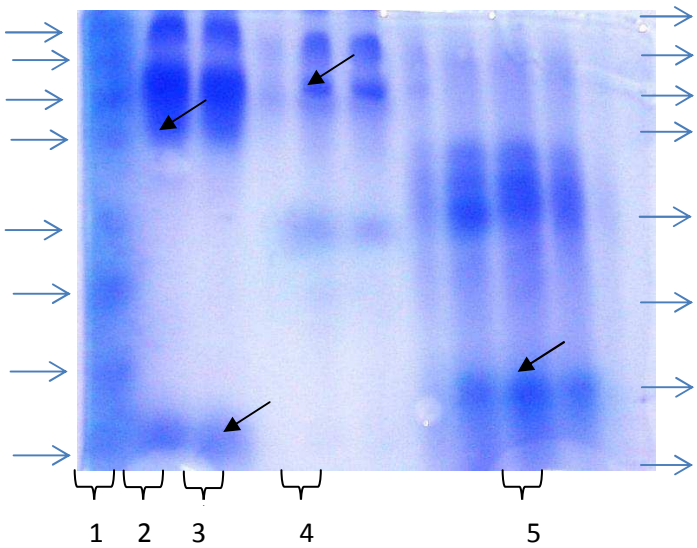

Figure № 1 : SDS-PAGE of hen eggs allergen proteins; Lane 1: standardprotein marker (200, 90, 65, 45, 30, 20 et 8 KDa); lane 2 :ovalbumin; lane 3 :ovomucoid; lane 4 : conalbumin; lane 5 : lysozyme.

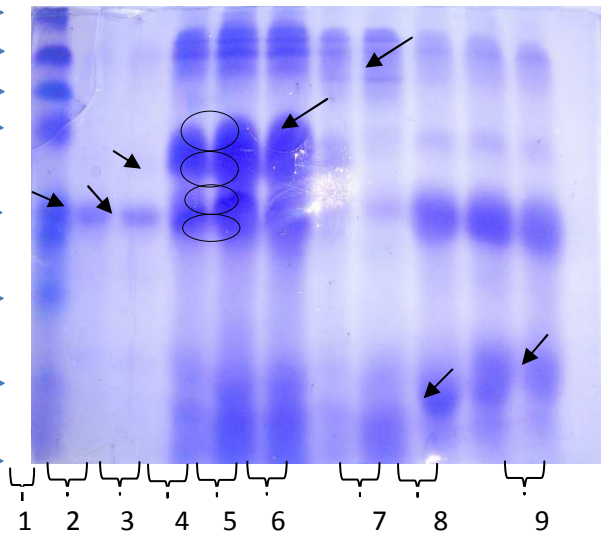

Figure № 2 : SDS-PAGE of cow s milk allergen proteins; Lane 1 : standard protein marker (200, 90, 65,45, 30, 20 et 8 KDa) ; lane 2 : αs1-casein; lane 3 : αs2-casein; lane 4 : κ-casein ; lane5 : whole casein; lane 6 : β-casein; lane 7 : serumalbumine; lane 8 : α lactalbumin; lane 9 : β-lactoglobulin.

The SDS-PAGE electrophoretic patterns of cow s milk and hen eggs allergen proteins are presented in fig. 1 and fig. 2, relative molecular weights (RMW) of ovalbumin, conalbumin, lysozyme, ovomucoïde, serum-albumin, β-lactoglobulin, αs1-casein, β-casein and k-casein are successively 50.22 KDa, 71.94KDa, 11.20 KDa, 11.25KDa, 74.13KDa, 14.92KDa, 27.94KDa, 45.89KDa and 41.68KDa. Results are in agreement with those reported by Chia-

Kai Su and Salem [18,19]. It is remarked that all the RMW are low, from 11.20 KDa to 74.13. it is known that all these proteins are monomeric except ovomucoid who is composed of three subunits, and who have the highest temperature denaturation, it is estimated that thermal resistance of trophallergens is not depending of the number of subunit (quaternary structure) but it is depending of the subunit itself (tertiary structure), its richness in disulfide bonds and its low RMW whom assure a geometric compact structure very resistant to aggressive treatments what helps to increase the allergenicity of these molecules.

Table № 1- physicochemical parameters of different protein allergens of cow s milk and hen eggs

Physicochemical parameters			Protein rate (%)	Carbohydrates rate (%)	Isoelectric point	Temperature Denaturation (°C)
Allergens proteins	Hen egg allergen	Ovalbumin	94.83	3.39	4.66	68.76
		Ovomucoïd	77.66	21.668	4.12	98.88
		Conalbumin	68.74	2.7	6.29	55.11
		Lysozyme	82.94	1.39	10.95	69.25
	Cow s milk allergen proteins	α-casein	78.52	1.63	4.63	60.76
		β-casein	78.51	1.92	4.61	59.27
		κ-casein	65.59	2.23	4.6	53.8
		Whole caseins	82.90	3.04	.63	52.6
		Serumalbumine	81.22	4.1	4	72.57
		β-lactoglobulin	89.57	4.1	4.73	76

Table № 2: physicochemical parameters of different protein allergens of cow s milk and hen eggs comparison

Physicochemical parameters	Statistics							
	Regression			Residual			Fobs	P
	df	SS	MS	df	SS	MS		
Protein rate (%)	9	220.0791	24.453	50	0.4107	0.0082	2976,90***	0,000***
Carbohydrate rate(%)	9	10543.56	1171.51	50	52.32	1.05	1119,64***	0,000***
Isoelectric point	9	5745.2	638.4	50	3456.5	69.1	9 ,23***	0,000***
Temperature denaturation (°C)	9	4044.37	449.37	50	63.23	1.26	355,35***	0,000***

****: Very highly significant differences .*

Tableau № 3: Relationships between physicochemical parameters of different protein allergens of cow s milk and hen eggs.

Variables		Probability	Correlation	coefficient	Regression	coefficient	Regression
X	**Y**	**p**	**r**	**R^2%**	**Y=a+bX**	**Fobs**	**Syx**
Isoelectric point	**Temperature denaturation (°C)**	NS 0.89	-0.051	/	/	/	/
Isoelectric point	**Carbohydrate rate (%)**	NS 0.409	-0.294	/	/	/	/
Isoelectric point	**Protein rate (%)**	NS 0.897	0.047	/	/	/	/
Temperature denaturation (°C)	**Carbohydrate rate (%)**	0,002**	0.841	70.80%	Td = 57,71 + 1,940 Tg	19,37 **	8.01254
Carbohydrate rate (%)	**Temperature denaturation (°C)**	0,002**	0.841	70.80%	Tg= - 19,70 + 0,3649 Td	19,37**	3.47524
Temperature denaturation (°C)	**Protein rate (%)**	NS 0.359	0.325	/	/	/	/
Carbohydrate rate (%)	**Protein rate (%)**	NS 0.93	-0.032	/	/	/	/

*NS: No significant differences, ** : Highly significant differences.*

Based on results concerning ovomucoid and b-lactoglobulin, it is revealed that when the protide is rich in cystein and tyrosin, have an acidic isoelectric point and low RMW and increasing amount of carbohydrate rate, it became more resistant to heat treatment.

Tableau № 4: Amount of amino acids in gramme per gramme of cows milk and hen eggs protidic extract determinated by HPLC

Amino acids in g/g of protidic extract										
Protéines Acideaminés	Ovalbumin	Ovomucoïd	Conalbumin	Lysozyme	αs 1-casein	β-casein	κ-casein	Whole caseins	β-lactoglobulin	Sérumalbumin
Aspartic acid	<0.003	<0.003	<0.003	<0.003	<0.003	≈0.01	<0.003	<0.003	<0.003	<0.003
Threonine	<0.003	<0.003	<0.003	<0.003	<0.003	<0.003	<0.003	<0.003	<0.003	<0.003
Serine	<0.003	≈0.01	<0.003	<0.003	<0.003	≈0.01	<0.003	<0.003	<0.003	<0.003
Glutamic acid	<0.003	<0.003	<0.003	<0.003	<0.003	≈0.01	<0.003	<0.003	<0.003	<0.003
Proline	<0.003	<0.003	<0.003	<0.003	<0.003	<0.003	<0.003	<0.003	<0.003	<0.003
Glycine	<0.003	≈0.01	<0.003	<0.003	<0.003	<0.003	<0.003	<0.003	<0.003	<0.003
Alanine	<0.003	<0.003	<0.003	<0.003	<0.003	≈0.01	<0.003	<0.003	<0.003	<0.003
Valine	<0.003	<0.003	<0.003	<0.003	<0.003	<0.003	<0.003	<0.003	<0.003	<0.003
Cysteine	0.0263	≈0.01	≈0.01	≈0.01	<0.003	≈0.01	0.011	0.013	0.076	0.095
Methionine	<0.003	<0.003	<0.003	<0.003	<0.003	<0.003	<0.003	<0.003	<0.003	<0.003
Isoleucine	<0.003	<0.003	<0.003	<0.003	<0.003	<0.003	<0.003	<0.003	<0.003	<0.003
Leucine	<0.003	<0.003	<0.003	<0.003	<0.003	<0.003	<0.003	<0.003	<0.003	<0.003
Tyrosine	0.0264	≈0.01	≈0.01	≈0.01	<0.003	≈0.01	0.011	0.021	0.0123	<0.003
Phenylalanine	<0.003	<0.003	<0.003	<0.003	<0.003	≈0.01	≈0.01	<0.003	<0.003	<0.003
Lysine	<0.003	<0.003	<0.003	<0.003	<0.003	≈0.01	<0.003	<0.003	<0.003	<0.003
Histidine	<0.003	<0.003	<0.003	<0.003	<0.003	<0.003	<0.003	<0.003	<0.003	<0.003
Arginine	<0.003	<0.003	<0.003	<0.003	<0.003	<0.003	<0.003	<0.003	<0.003	<0.003
Tryptophane	-	-	-	-	-	-	-	-	-	-

CONCLUSION

Ovomucoid is the most allergic egg protein, very resistant to heat, temperatures denaturation of conalbumin, ovalbumin and lysozyme are successively 55.11°C and69,25°C. B-lactoglobuline and bovine serumalbumine are denaturated successively at 72.57°C and 76°C. After their isolation caseins become more sensible to heat treatments. It is revealed the existence of thermorsistant and thermolabile allergens. Most of allergen proteins have a glycan moiety, acid isoelectric point and they are all of low molecular weight. Carbohydrate portion protect these molecules from heat treatments. An important abundance of hydrogen bonds, hydrophobic interactions and disulfide bonds confer to the molecule a compact structure resistant to the unfolding of spatial arrangement of the peptidic chain and increase the thermal stability. It is also concluded that Trophallergens characterized by an acid isoelectric point, low relative molecular weight, high content of carbohydrate and rich of cystein and tyrosinresidus are necessary thermostable. When trophallergens have a basic or near of neutrality isoelectric point, an unimportant content of glycan portion and when they are of low relative molecular weight and poor in cystein and tyrosin, they are necessary thermolabile.

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