

# Alfalfa Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase: Extraction, Biochemical Characterization and Proteolytic Hydrolysis Optimization Process for Plausible Biotechnological Tools

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## Abstract

Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) is the major enzyme assimilating CO<sub>2</sub> into the biosphere. In this work, we report the extraction, the purification of the Alfalfa Rubisco. Indeed, the purification steps of the last one lead to the purified protein composed of two subunits (13 kDa and 55 kDa) as indicated in SDS-PAGE. The Rubisco proteolytic process via proteinase-K was optimized. The Box-Behnken Response Surface Methodology was applied to optimize three factors for increasing peptide production. So, the proteinase-K concentration (6, 13 and 20 mg/mL), the reaction incubation time (6 h, 8 h and 10 h) and the reaction temperature (30°C, 43°C and 56°C) were selected for the experimental design. A best condition of 20 mg/mL of proteinase-K, 10 h of time incubation, and 30°C of temperature treatment resulted in a 79.83%. This yield was 2.66 times higher than the one achieved before optimization. The resulting hydrolysate was injected in gel filtration column coupled to HPLC giving rise to four fractions (F1, F2, F3 and F4) eluted respectively after 12.54 min, 12.47 min, 12.44 min and 12.60 min. Also, the ROS scavenging system was monitored like the CAT, SOD and also the DPPH capacity. Eventually, those findings will give birth to the opportunity to look for plausible biotechnological investigations.

**Keywords:** Alfalfa; Rubisco purification; Hydrolysis process; Proteinase-K; Rubisco modelling; Biotechnological tools

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## Introduction

Proteins are widespread worldwide, they are indispensable for humans and animals as they are essential for covering amino acid requirements [1,2]. Animal meat is known as the primary source of protein for human nutrition. However, following the strong global population growth, several studies and investigations have shown that meeting the need for animal protein seems particularly difficult in the future [3]. Therefore, it is necessary to double the meat production [4] but this solution will cause an increased demand for animal feed as it consumes approximately ¼ of the world's produced biomass [5] and subsequently, heavily affects the availability of vegetation and arable soil which are limited. For this reason, we are looking for new sources of protein to replace those that are no longer effective and which

are cheaper, healthier, more nutritious and friendlier for the environment [6]. Pulses and cereals are the non-animal source for humans [7] and in recent years, plant leaves are considered to be the main source of vegetable protein [8-10]. Alfalfa (*Medicago sativa* L.) ranks among the most important vegetable protein sources in the world. It is a forage legume grown on 33 million hectares in the whole world [11]. It is known for its high forage quality, its contribution in nitrogen fertilization and its high protein content (enrichment of animal feed). Thus, in addition to the use in the feeding of cattle, alfalfa enters into human nutrition like sprouted seeds and juices; this is due to its valuable protein considering alfalfa as a source of secondary metabolites for the agricultural and pharmaceutical industry [12]. This legume was

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characterized by its protein-rich leaves and in particular Rubisco that is the most abundant enzyme in our planet [13] and it constitutes about 65% of the soluble alfalfa leaf protein [10]. This intake, essential amino acids for human health, mineral salts and macro and micro nutrients [14]. Also, its antioxidant capability and its richness in phytoestrogens, saponins and vitamins allow bioactive molecule has been extracted from different plant parts (roots, stems, flowers and leaves) [15,16] and it is considered to be a protein complex having a molecular weight of 560 kDa and consisting of eight copies of large protein chain (55 kDa) [17] and eight copies of a small protein chain (14 kDa) [18].

It is crucial and so important to signal that Rubisco seems to be a new potential source of bioactive peptides [16]. Indeed, the goal of this work is to extract Rubisco from Gabesian alfalfa leaves (southern Tunisia) that was the most salt tolerant one among 12 accessions lines studied by our group (data in publication) and there were different methods allow the extraction of concentrated total proteins [19,20]. In our study we followed methodology described by Kobbi S, et al. [10] for the Rubisco extraction and purification from Alfalfa leaves. Further, bioactive peptides from extracted Rubisco were done using hydrolysis by proteinase-K. This methodology reduces complexity, cost and environmental problems associated with conventional industrial processes.

## Materials and Methods

### Biological materials

50g of Gabesian alfalfa leaves (the region of Limaoua) were used. The leaves were harvested, crushed and cold pressed. The resulting paste was dried in a furnace at (37°C) to obtain an heterogeneous dried alfalfa powder. Then, the last one was ready for Rubisco purification. This method is the dehydration process as described by Incamps et al. [21].

### Extraction and purification of Rubisco

After alfalfa leaves dehydration process, 10 g of powder with high protein content was obtained and the protein extraction

and purification were carried out as indicated in [10]. In order to isolate the soluble fraction, 10g of the alfalfa powder was solubilized in 100 mL of distilled water at pH 10 by the addition of NaOH (0.1 M). A first centrifugation was made at 10000xg for 20min at 4°C to eliminate the green protein and all debris. The supernatant containing Rubisco was recovered and the pellet was centrifuged again to retrieve the second supernatant. After four successive centrifugations at pH 10, four solutions were summed up to recover all Rubisco. Then, proteins were precipitated by the acidification of the mixture at pH 3 using 10 mM of chloridric acid (37% PA-ACS-ISO, purchased from Panreac) to precipitate the proteins. This was followed by a last centrifugation at 10000xg for 20 min at 4°C. The precipitate was recovered and the Rubisco was then concentrated by glacial acetone (analytical grade, ACS, Reag. Ph Eur, purchased from Scharlau under matricule number of AC03111000).

### Protein assay and electrophoretic mobility

The protein content was determined using the Biorad-Bradford assay with absorbency reading at 595nm. The Bovine Serum Albumin (BSA) was used as protein standard [22]. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) was carried out in 15% polyacrylamide gel following the standard procedure by Laemmli and Favre [23].

### Optimization of Rubisco hydrolysis

The hydrolysis was carried out by mixing 1mL of the purified Rubisco with proteinase-K. Then the solution was hydrolyzed in a water bath [24] and the reaction was stopped by ice. The influence of temperature, time of incubation and also proteinase-K concentration was studied using the 'one variable at a time' method. The Box-Behnken Response Surface Methodology was applied to optimize the three factor levels for increasing the efficiency of peptides production. These three independent parameters were taken at three levels (-1, 0, +1) and 15 assays were performed as demonstrated in **Table 1**.

**Table 1** The matrix illustrating the conditions that were used to optimize the hydrolysis of the Rubisco by protease-K.

Run No.	[Protease-K] (mg/mL)		Time(h)		Temperature (°C)		Yields (%)
	Actual value	Coded value	Actual value	Coded value	Actual value	Coded value	
1	6	(-1)	6	(-1)	43	(0)	60.55 ± 0.11
2	6	(-1)	10	(+1)	43	(0)	69.15 ± 0.25
3	20	(+1)	6	(-1)	43	(0)	72.75 ± 0.19
4	20	(+1)	10	(+1)	43	(0)	67.22 ± 0.15
5	13	(0)	6	(-1)	30	(-1)	72.97 ± 0.23
6	13	(0)	6	(-1)	56	(+1)	54.35 ± 0.12
7	13	(0)	10	(+1)	30	(-1)	74.62 ± 0.18
8	13	(0)	10	(+1)	56	(+1)	60.40 ± 0.21
9	6	(-1)	8	(0)	30	(-1)	71.32 ± 0.27
10	20	(+1)	8	(0)	30	(-1)	70.25 ± 0.19
11	6	(-1)	8	(0)	56	(+1)	55.75 ± 0.14
12	20	(+1)	8	(0)	56	(+1)	71.12 ± 0.24
13	13	(0)	8	(0)	43	(0)	64.00 ± 0.22
14	13	(0)	8	(0)	43	(0)	64.15 ± 0.29
15	13	(0)	8	(0)	43	(0)	64.97 ± 0.27

This hydrolysis was done at different temperatures (30, 43, and 56°C), at different enzyme concentrations (6, 13, and 20 mg/mL) and at different times (6, 8, and 10 h) to investigate the kinetics hydrolysis of Rubisco.

The mean values (n=3) of the yield were considered as responses. The yield was fitted according to the second-order polynomial model:

$$Y = a_0 + \sum_{i=1}^n a_i X_i + \sum_{i=1}^n a_{ii} X_i^2 + \sum_{i=j}^{n-1} \sum_{j=i+1}^n a_{ij} X_i X_j$$

In this equation, Y is the production yield,  $a_0$  is the intercept value,  $a_i$  is the linear value,  $a_{ii}$  is the second order value,  $a_{ij}$  is the interaction term and  $X_i$  is the independent parameters. The response surface diagrams were shown using the SPSS software (IBM SPSS version 21).

### DPPH radical-scavenging assay

DPPH radical-scavenging activity of the alfalfa crude was determined as described by Bersuder et al. and Hsouna et al. [25,26]. To do this, 500 µL of the sample was mixed with 500 µL of 99.5% ethanol and 125 µL of 0.02% DPPH in 99.5% ethanol. Then the mixture was kept in the dark room for 60 min, and the reduction of DPPH radical was measured at 517 nm. The control was conducted in the same manner; except that distilled water was used instead of sample and Butylate-dihydroxy-toluene (BHT) was used as standard. The DPPH activity was calculated as follows:

$$(\%) = [1 - (\text{Absorbance of sample} / \text{Absorbance of negative control})] \times 100.$$

### Statistical analysis

All data were submitted to Analysis of Variance (ANOVA) and differences between means were evaluated by Duncan's Multiple Range Test. The data were analyzed using the Statistical Package for the Social Sciences (SPSS) version 10.0 (Chicago, Illinois, USA). Differences were considered significant at  $p < 0.05$

## Results and Discussion

### Production and purification of Rubisco

Rubisco is a bioactive molecule that has been labeled the most abundant and largest protein on earth [10,27]. In our study, the dried green powder was prepared from alfalfa leaves in order to extract and purified Rubisco at homogeneity. Indeed, and as indicated in **Figure 1**, after three centrifugation of alfalfa green juice, we recovered the major Rubisco fraction. Then, the Rubisco solution was centrifuged and the recuperated supernatant was precipitated using the acetone concentration protocol. Proteins content of the purified Rubisco was estimated to 40 mg/mL.

### SDS-PAGE and *in silico* analysis of the Rubisco

The Rubisco obtained from the green juice was analyzed by SDS-PAGE on 15% (w/v) to confirm their purity. Indeed, **Figure 2** confirms that the studied protein was purified at homogeneity and is composed of large subunit (L) possessing an apparent

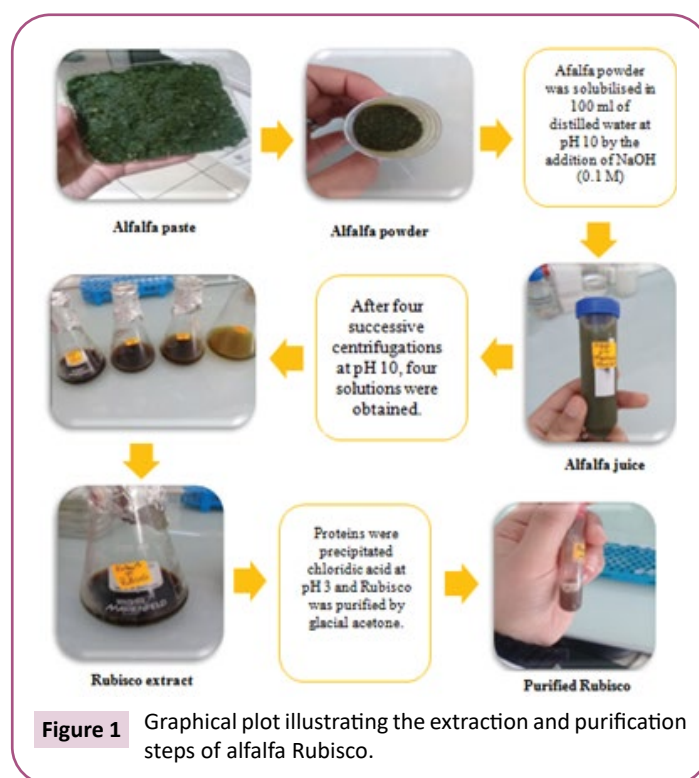
molecular weight of 55 kDa and small subunit (S) possessing an apparent molecular weight of 13 kDa. Quaternary structural studies have previously demonstrated that native Rubisco is a protein consisting of eight small and eight large subunits [28,29].

On the same case, **Table 2** shows that Rubisco is composed of 569 amino acids: 446 amino acids in the large subunit which their molecular weight is about 55 kDa and 123 amino acids in the small subunit which their molecular weight is about 13 kDa. The theoretical isoelectric point is computed to be 6.6 to 6.9 for the subunit L and 7.45 for the subunit S (**Table 2**).

### Optimization of the proteolytic Rubisco hydrolysis

The Box-Behnken Response Surface Methodology was monitored in order to determine the effects of the three selected variables (the reaction temperature (°C); the reaction incubation time (h) and the proteinase-K concentration (mg/mL) on peptide productions. The experimental results were summarized in **Table 1**. The results could be elucidated by the following non-linear regression model using the SPSS software (IBM SPSS version 21).

$$Y = 111.537 - 0.699 \times X_1 - 0.908 \times X_2 - 1.614 \times X_3 + 0.047 \times X_1 \times X_1 + 0.190 \times X_2 \times X_2 + 0.003 \times X_3 \times X_3 - 0.252 \times X_1 \times X_2 + 0.045 \times X_1 \times X_3 + 0.042 \times$$



**Figure 1** Graphical plot illustrating the extraction and purification steps of alfalfa Rubisco.

**Table 2** Illustration of some of the Rubisco *in silico* parameters such as the amino acid composition, the molecular weight and also the theoretical isoelectric point.

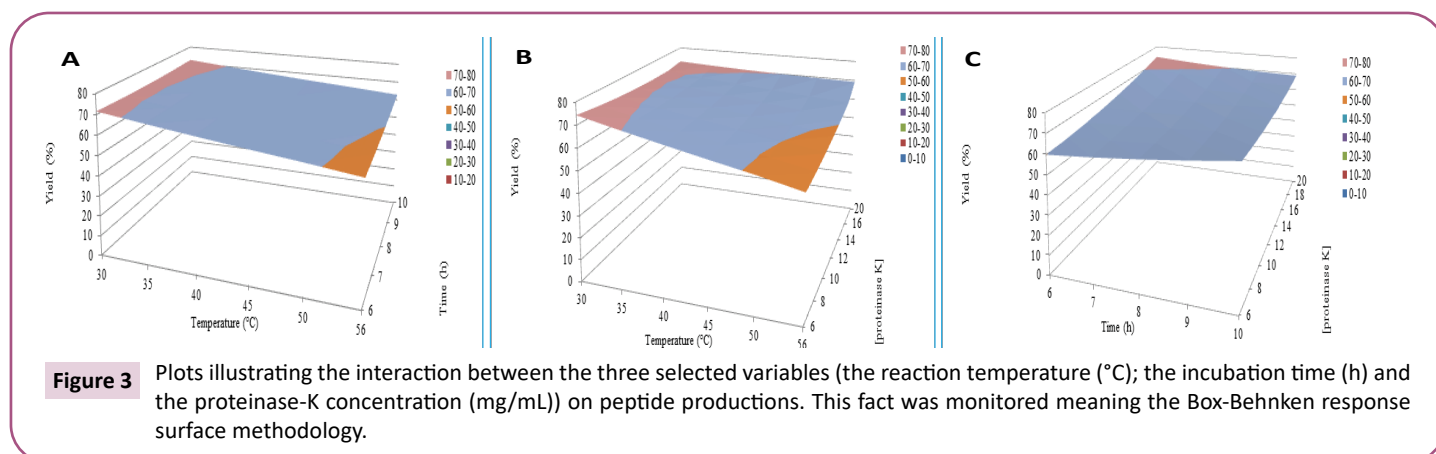
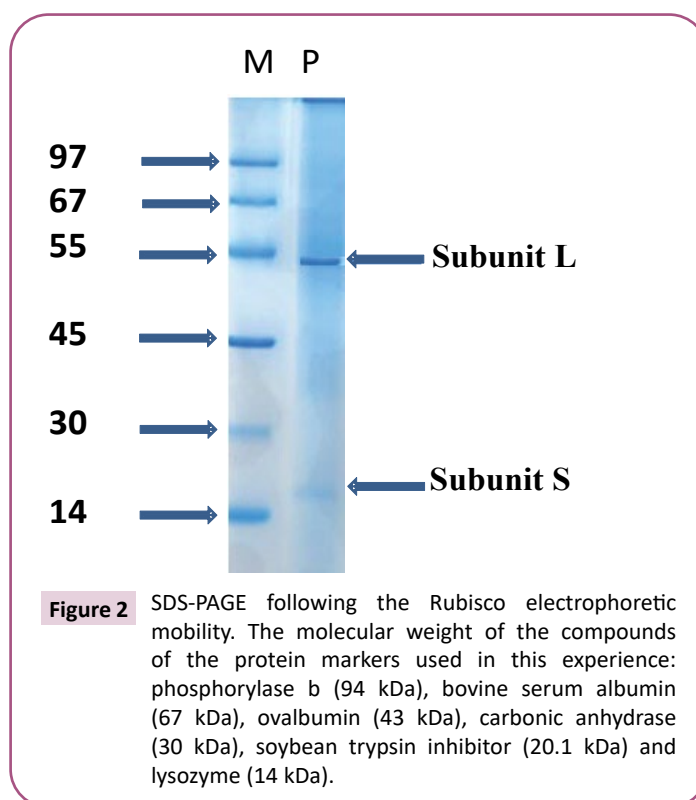
Rubisco characteristics	Large subunit	Small subunit
Amino acid composition	446	123
Molecular weight	55 kDa	14 kDa
Theoretical isoelectric point	6.6 to 6.9	7.45

$$X_2 \times X_3$$

In this equation, Y refers to peptide production yield (%),  $X_1$  to the proteinase-K concentration (mg/mL),  $X_2$  to the reaction incubation time (h), and  $X_3$  to the reaction temperature (°C). This yield model was highly significant ( $p < 0.01$ ) with an important determination value of ( $R^2 = 0.917$ ) confirming that 91.7% of the inconsistency in the response could be explained by the above model. This equation was then investigated by means of "F" statistical analysis by ANOVA to test the goodness of fit. The "F" value (111.396), with a very low probability value, ( $p < 0.001$ ) demonstrated that this equation was highly significant. Indeed, the higher of the "F" value is the more significant deduced model becomes. Then, the adjusted coefficient of determination ( $Ra^2 = 0.913$ ) proved a better consistency and precision of the approved assays [2,30].

The studies of the following equation also displayed that peptide production depended ultimately on the linear and quadratic terms of the three selected parameters. In this model, the interactions between temperature with, respectively, proteinase-K concentration ( $X_1, X_3$ ) and reaction incubation time ( $X_2, X_3$ ) show a positive effect on hydrolysis yield, whereas proteinase K concentration and reaction time of incubation ( $X_1, X_2$ ) shows a negative effect. The interactions between the parameters could be well visualized by plotting the 3D response surface designs. Consequently, the response plots were done by varying the two variables while the third one was kept at its middle level as indicated in **Figure 3**.

These surface response plots exhibited a significant curvature in curves suggesting that these variables were interdependent at different levels. The peptide production level increased when the

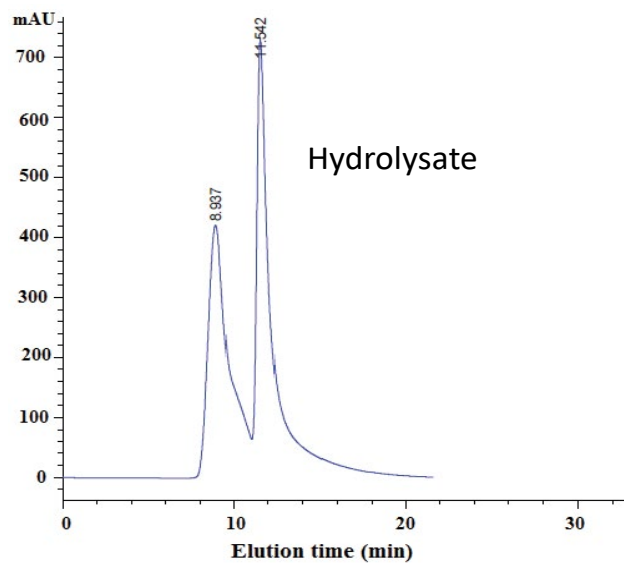


proteinase-K content increased but it decreased at a high level of temperature. Additionally, the optimal conditions were found in the high level of the reaction incubation time values. The validity of the polynomial equation was verified by doing the optimum conditions: 20 mg/mL of proteinase-K concentration, a 10 h of reaction time incubation, and 30°C of incubation temperature. The predicted peptide production was 79.83%. This production was validated through three experiments at an optimal condition whose results were 81%, 82%, and 83%. The high correlation

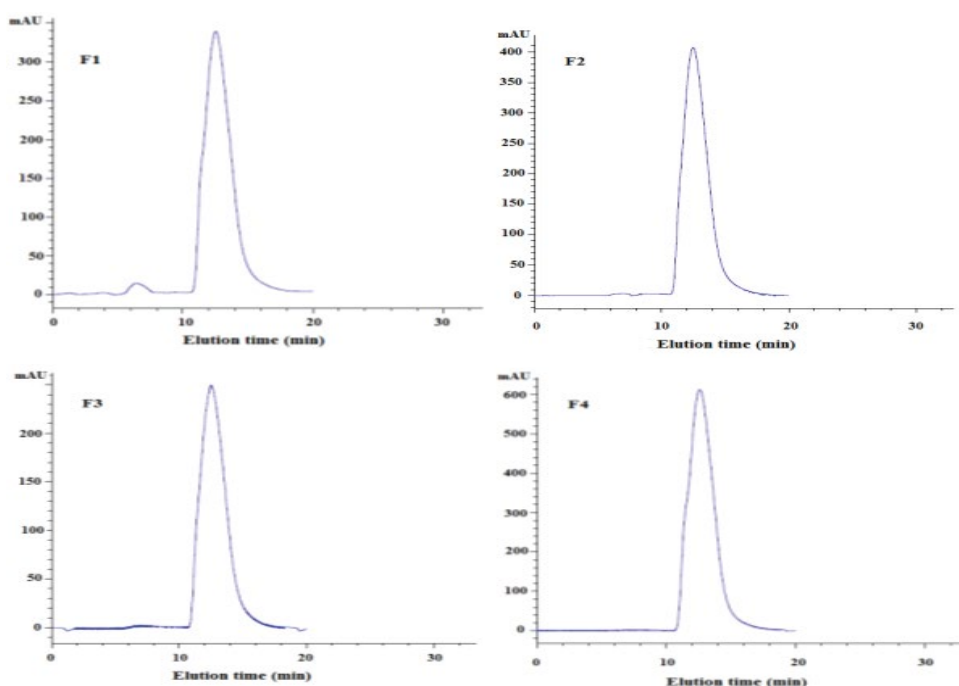
between the predicted and the experimental values displayed the validity of the response fitted model. The peptide yield was approximately 2.66 times higher than the one obtained before optimization. Certainly, controlling the physical parameters of the hydrolyzing dramatically enhanced the peptide production.

### Chromatographic studies and characterizations of the hydrolysate

After hydrolyzing the prepared Rubisco solution meaning



**Figure 4** Chromatographic profile illustrating the injection of the hydrolysate Rubisco in gel filtration column coupled to HPLC system.



**Figure 5** Chromatographic profile illustrating the injection of the four fractions eluted from the hydrolysate Rubisco solution after their treatment with gel filtration column coupled to HPLC system.

**Table 3** Illustration of the assessment of the CAT, SOD and DPPH levels of the crude extract and the described eluted fractions (F1, F2, F3 and F4).

	CAT (U/mg protein)	SOD (U/mg protein)	DPPH (%)
Crude Extract	79,73	1289,13	35,34
F1	18,4	30,54	78,87
F2	2,3	26,15	56,89
F3	11,5	43,65	49,13
F4	57,5	19.35	37,93

proteinase-K, we proceed to the separation of the potential released peptides. In fact, the alfalfa Rubisco was loaded to reversed-phase high performance liquid chromatography analysis in order to evaluate its purity and the potential effects of the extraction process on protein quality. To do this, the hydrolysate was injected to gel filtration column coupled to HPLC system. The result is followed in **Figure 4** illustrating the chromatographic profile.

The presence of two peaks at an elution time of 9 min and 11.5 min was taken as an indicator for the presence of Rubisco in the sample [31]. Indeed, four fractions were recuperated (named F1, F2, F3 and F4, respectively) and re-injected on the same column–HPLC system, as shown in **Figure 5**. The described eluted fractions will be used as plausible tools for some of biotechnological applications related to para-pharmaceutical and agrofood fields.

### Radical-scavenging system assessment

For more characterizations, the separated fractions were used to measure some physiological parameters such as CAT, SOD and DPPH. Indeed, those parameters were monitored in the case of the crude extract and also in the case of the peptide fractions. Indeed, **Table 3** follows that the CAT activity level seems to be the same on the crude extract and the F4 fraction. This finding pleads for the fact that this enzymatic activity is encoded, for probably, by F4. In another hand, the SOD activity monitored on the crude extract is not arised in any one of those described fractions (F1, F2, F3 and F4). In addition, the DPPH appears in all the eluted fractions and we can't attribute this physiological parameter to one of them. Eventually, the described eluted fractions are able to be used as effectors in various fields.

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According to Nelson SK, et al. [32], a study was done on healthy human subjects whom were injected by a mixture of extracts of five medicinal plants after induction of SOD and CAT. This experiment was based on the results made on rodents with evidence of decreasing lipid peroxidation. Findings showed that modest induction of the catalytic antioxidants SOD and CAT may be a much more effective approach than supplementation with antioxidants (such as vitamins C and E) that can, at best, stoichiometrically scavenge a very small fraction of total oxidant production. In addition to that, others findings demonstrate that the altered energy balance in High fat diet fed mitochondrial catalase mice protected them diacylglycerol accumulation, protein kinase C activation and impaired muscle insulin signaling [33]. According to those data, we will be look for the plausible use of the catalase capability of our biological matrix as a pharmaceutical or/and para-pharmaceutical product.

## Conclusion

Based on those findings, we conclude that the Alfalfa Ribulose-1,5-bisphosphate carboxylase/oxygenase will be an excellent candidate protein in the future so as to contribute to the improvement of various biotechnological processes related to the agro-food industry as additive and also for their capability as clinical and also as pharmacological effector.

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