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Leaf Protein Availability in Food: Significance of the Binding of Phenolic Compounds to Ribulose-1,5-Diphosphate Carboxylase

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The binding of phenolic compounds to ribulose-1,5-diphosphate carboxylase (RubisCO) is known to give rise to some digestive problems in human beings. In fact, the biological value of protein and hence the Protein Efficiency Ratio and Net Protein Utilization decrease drastically. For this reason the binding of phenolic compounds (e.g. rutin and chlorogenic acid) to ribulose-1,5-diphosphate carboxylase (RubisCO) was studied by means of ultrafiltration techniques in order to better elucidate the nature of this interaction and the factors influencing it in an attempt to limit or avoid it. RubisCO behaviour was also compared with that of Bovine Serum Albumin. A multivariate approach was used to determine the most influencing variables and their effects on binding. A classical binding study with the aim of determining the binding stoichiometry was also carried out. pH was found to be the most important variable affecting the binding of rutin to RubisCO as well as rutin to Bovine Serum Albumin while contact time became relevant when operating in sub-alkaline pH conditions. Classical binding analysis was carried out at pH 7.0 to 7.3 by both direct partition and diafiltration methods. A total number of five binding sites was determined, with two kinds of binding mechanisms, one of which was hydrophobic. The diafiltration method can be considered a useful tool when high affinity interactions are studied; RubisCO protein stability was disturbed by stirring, but this allowed an increased affinity of aggregated RubisCO to chlorogenic acid to be noted. This might have important consequences on RubisCO extraction technology since the most critical phase of phenolic contamination is the crystallization–precipitation step.

Introduction

During the last 20 years, leaf concentrates and isolates have been considered to be an important nonconventional protein source due to their balanced amino acid profile and functional properties and to the relatively simple extraction technology used. Bray (1), Fiorentini and Galoppini (2), Carlsson (3) and Montanari (4) have reviewed this topic.

The present uses of leaf protein extraction techniques in developed countries are: optimization of fodder crop fractionation in order to obtain low purified concentrates for feeding; improvement of leaf juice fractionation to obtain highly purified fractions for food and clinical-pharmaceutical utilization, i.e. not only proteins but also chlorophyll, carotenoids, polyphenols.

As far as the protein fractions are concerned, since the 1980s scientists have concentrated their interest on the enzyme ribulose-1,5-diphosphate carboxylase, no longer only from a biochemical point of view, but also as a likely commercial protein product due to its balanced amino acid profile and to its ubiquitousness and abundance.

The enzyme ribulose-1,5-diphosphate carboxylase is also known as F_1P (Fraction 1 Protein) or RubisCO; the main interest in extracting RubisCO from the tobacco plant is due to its unique property to crystallize into virtually pure crystals in low ionic strength conditions. This property, first discovered in 1971 by Kawashima and Wildman (5) and subsequently applied to medium scale extraction procedures by Lowe (6), allows easy utilization of this enzyme in the food industry as an ingredient in different food preparations.

The suggested standard procedure, where gel filtration using Sephadex provides polyphenol removal and buffer exchange to low ionic strength, is relatively simple and widely used in laboratories; a technological process, more suitable for large scale extraction, has been used at the University of Perugia on several plant species (4).

In the technological extraction procedure the isolation of F_1P is obtained via precipitation near the isoelectric point (7). In such pH conditions tobacco F_1P does not form pure crystals but a mixture of crystals and amorphous isoelectric proteins; besides, tobacco is the only species from which crystalline RubisCO has been so far obtained in sufficient amounts.

Purity is essential for each large-scale process of extraction and fractionation, especially when a product of high biological value is considered. Analyses of the presence of heavy metals and mutagenicity have already been carried out (8–10) as well as the purification of nicotine residues by supercritical CO_2 extraction

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(11); however there is still some contamination from phenols and Fantozzi *et al.* (11) showed that supercritical CO_2 extraction is not effective in their removal.
Considering that the biological value of F_1P, PER (Protein Efficiency Ratio) and NPU (Net Protein Utilization) decrease in the presence of phenols (12), resulting in less effective digestion and assimilation by humans, the present study was conducted to investigate the interaction between RubisCO extracted from spinach and tobacco and simple phenols (rutin and chlorogenic acid), with the aim of better understanding the nature of this interaction, the factors influencing it and attempts to limit or avoid it.
Bovine Serum Albumin was used as a reference and cross-control with other authors.

Materials and Methods

Reagents

Ribulose-1,5-diphosphate carboxylase (RubisCO) was extracted from leaves of *Spinacia oleracea* L. according to the protocol described in the section *RubisCO extraction*. The spinach was purchased from a local market.
Ribulose-1,5-diphosphate carboxylase (RubisCO) was extracted from *Nicotiana tabacum* L. plants, according to the protocol described in the section *RubisCO extraction*. The variety Bright Cospaia White Flower, grown in a greenhouse at the Botanical Garden of University of Perugia (seedlings supplied by Istituto di Miglioramento Genetico Vegetale, University of Perugia), was used.
Trihydrated rutin (BDH, art. 44252 3F, Milano, Italy).
Chlorogenic acid (SIGMA, Chemical company, art. C 3878, St. Louis, U.S.A.).
Bovine Serum Albumin (BSA) Fraction V, (SIGMA Chemical company, art. A 4503, St. Louis, U.S.A.).
Sephadex G-50 (coarse) and Sephadex G-75 (Pharmacia Fine Chemicals, Uppsala, Sweden).
Phosphate buffer, mercaptoethanol, glycerol and Tris-HCl buffer, were from Carlo Erba (Milano, Italy); EDTA, NaOH, borate buffer and sodium azide were from BDH (Milano, Italy); for the protein assay BIO-RAD Coomassie Brilliant Blue G-250 (BIO-RAD, Milano, Italy) was used.

Apparatus and other materials

Cellulose acetate syringe filters LIDA SPA-5002 porosity 0.22 μm and SPA-5003, 0.45 μm (LIDA, Kenosha, WI, U.S.A.).
Disposable centrifuge ultrafiltration units Centrifree CL (Millipore, Milano, Italy) with a 10,000 cut-off.
Ultrafiltration stirred cell (50 mL) Amicon model 52 with diafiltration accessories.
Ultrafiltration cellulose triacetate discs SM 14529 (cut-off 10,000) (Sartorius, Firenze, Italy and Hayward, CA, U.S.A).

RubisCO extraction

RubisCO was extracted from spinach according to the procedure of Jones and Mangan (13) with some minor modifications. A 35 cm column (i.d. 5 cm) packed with Sephadex G-75 and equilibrated with 50 mmol/L, pH 7.5 phosphate buffer, containing 0.1 mmol/L of mercaptoethanol was used to separate the proteins from pigments and salts.
The first eluted fraction (about 60 mL) was collected and stored at –20°C with 200 mg/mL of glycerol until use.
RubisCO from tobacco was obtained according to the procedure described by Kung *et al.* (14): a 50 cm column (i.d. 10 cm) packed with Sephadex G-50 (coarse) equilibrated with 25 mmol/L, pH 7.4 Tris-HCl buffer, containing 0.2 mmol/L of EDTA was used to separate the proteins from pigments and to exchange the buffer to low ionic strength. Crystallization was completed during storage overnight at 4°C. Crystals were washed twice with the same buffer and stored at 4°C in the elution buffer with 200 mg/L of sodium azide until use.

Binding studies

Among the various methods available for binding studies (fluorimetry, calorimetry, ultrafiltration, equilibrium dialysis, gel filtration, etc.) (15), ultrafiltration was chosen because data collection is rapid and it requires minimal sample preparation; this is particularly important for studying sensitive macromolecules. The reliability of this method was theoretically and experimentally demonstrated by Sophianopoulos *et al.* (16,17).
There are two main ultrafiltration techniques: direct partition and diafiltration. The former consists of preparing several mixtures of ligand and protein solutions, each at known concentrations, leaving them to reach binding equilibrium and then subjecting them to ultrafiltration through a selective membrane: the ligand concentration measured in the permeate represents the free ligand; the bound ligand is therefore calculated by difference from the total amount of the ligand.
The latter method is slightly more complex, but it can provide, under appropriate conditions, more information on binding behaviour from a single experiment; it is particularly recommended for the determination of binding isotherms. The protein solution is contained in an ultrafiltration stirred cell, while the ligand solution (stock solution) is gradually fed under pressure into the cell from a reservoir (wash-in technique); the permeate is removed from the cell at the same rate so that the volume put into the cell, and therefore the protein concentration, remain constant; the permeate is collected in small aliquots (1/5 to 1/10 the cell volume) and

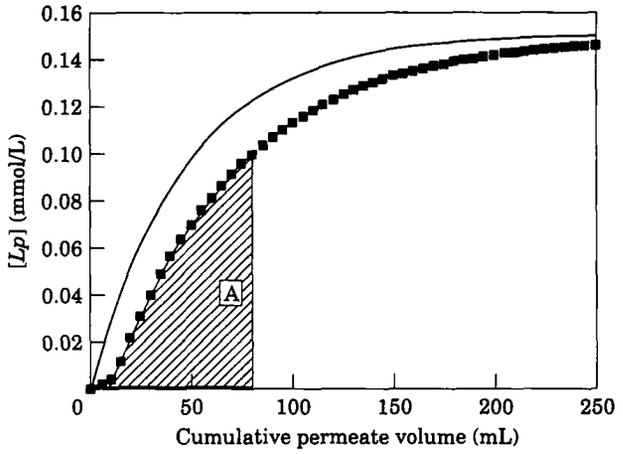

Fig. 1 Comparison between ideal (——) and experimental (actual with protein = ■) dilution curves in a typical diafiltration binding experiment. The shaded area under the curve (A) represents the total amount of ligand collected in the permeate up to that cumulative permeate volume; $[Lp]$ = permeate ligand concentration. Redrawn from Cheryan and Saeed (28)

the ligand concentration is determined. If no ligand binds to the protein, the concentration measured in the permeate depends on the stock solution concentration and the cumulative permeate volume only; however, when protein binds to the ligand, the ligand concentration in the permeate is lower than theoretically expected. A visual comparison between plots of theoretical and actual ligand concentrations in the permeate *vs.* the cumulative permeate volume gives an estimate of the extent of the binding. (**Fig. 1**).

The scheme of the apparatus used in the present study is shown in **Fig. 2**.

During the diafiltration run, whose dynamics were discussed by Cheryan (18), the instantaneous ligand concentration in the permeate is determined by the following equation:

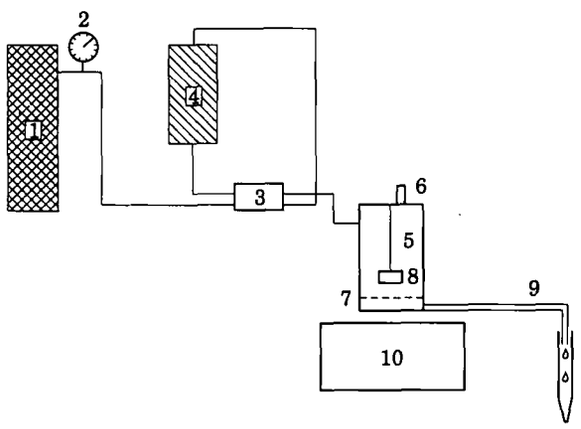

Fig. 2 Scheme of the continuous ultrafiltration assembly used in the binding studies. Key: 1 = Nitrogen cylinder; 2 = Pressure gauge; 3 = Three-way valve; 4 = Reservoir; 5 = Stirred cell; 6 = Pressure-relief valve; 7 = Membrane; 8 = Stirring bar; 9 = Permeate outlet; 10 = Magnetic stirrer

$$\ln \frac{[Lr]}{([Lr] - [Lp])} = (Vp - V') / Vc \qquad \text{Eqn [1]}$$

where: $[Lr]$ = ligand concentration in the reservoir; $[Lp]$ = ligand concentration in the permeate; Vp = cumulative volume of the permeate; Vc = volume of the solution in the cell; V' = apparent void volume of the system.

A linear transformation of Eqn [1] can be obtained using decimal logarithms so that a plot of log $[Lr]$ / $([Lr] - [Lp])$*vs.* Vp would give a straight line, with the intercept on the x-axis providing an estimate of the apparent void volume and the slope being equal to $1 / (2.303 \cdot Vc)$.

In order to obtain the desired binding parameters a mass balance of the quantities that have flowed through the ultrafiltration cell should be carried out:

Moles of ligand in (M in) = Moles of ligand out in the permeate (M out) + Moles of ligand accumulated in the cell (Mc). [Eqn 2]

Moles of ligand accumulated in the cell (Mc) = Moles bound to the protein (Mcp) + Moles of free ligand in the cell (Mcf). [Eqn 3]

If: Vr = volume of ligand fed into the cell from reservoir; $[Lr]$ = ligand concentration in the reservoir; Vp = cumulative volume of the permeate; $[Lp]$ = ligand concentration in the permeate; Vc = volume of protein solution in the cell.

We can assume that:

(a) M in = $Vr \cdot [Lr] = Vp \cdot [Lr]$ (because the permeate is removed at the same rate of ligand feed);
(b) Mcf = $Vc \cdot [Lp]$ (if binding of ligand to the membrane can be excluded);
(c) M out = A = Area under the diafiltration curve (in the presence of the protein).

The area can be determined using a computer program able to calculate a polynomial regression from raw data, using the following equation:

$$y = A_0 + A_1 x + A_2 x^2 + A_3 x^3 + \ldots \qquad \text{Eqn [4]}$$

where $y = [Lp]$ and $x = Vp$. Such a polynomial equation can be integrated to give A:

$$A = A_0 x + A_1 x^2/2 + A_2 x^3/3 + A_3 x^4/4 + \ldots \qquad \text{Eqn [5]}$$

The moles of ligand bound to the protein in the cell can be calculated by expressing the quantities in equation M in = M out + Mcp + Mcf with the experimentally known values, obtaining:

$$Vp \cdot [Lr] = A + \text{Mcp} + Vc \cdot [Lp] \qquad \text{Eqn [6]}$$

therefore:

$$\text{Mcp} = Vp \cdot [Lr] - A - Vc \cdot [Lp] \qquad \text{Eqn [7]}$$

Dividing Mcp values so obtained (for each experimental point) by the moles of protein contained in the cell the molar binding ratios r are obtained.

The two ultrafiltration techniques above described

were applied to two different strategies and for different purposes: (a) a multivariate data collection strategy in order to study the influence of various factors on the extent of binding (direct partition technique); (b) a classical binding study which was carried out in order to determine binding parameters (direct partition and diafiltration techniques).
The first experiment was carried out using rutin as ligand and both BSA and spinach RubisCO as proteins. In the second, RubisCO from tobacco and chlorogenic acid were used.

Multivariate approach: fractional factorial design

A preliminary experiment was carried out on the BSA-rutin system, in order to determine the most influential explanatory or causal or independent variables among temperature, contact time, ligand concentration, pH and ionic strength (μ) on the extent of binding (response). A fractional factorial design (FFD), in which these five causal variables were tested in eight experiments, was used: the formula that gives the number of experiments in an FFD is 2^{n-k}, where n represents the number of variables under investigation and k represents the fraction of the experimental design; substituting values it becomes $2^{5-2}=8$. The above-mentioned variables were used at two levels, afterwards denoted with the symbols +1 and −1 (see **Table 1** for details on the variable values).
Solutions were prepared in appropriate buffers (acetic acid / sodium acetate 0.1 mol/L, pH 5.0, and phosphate buffer made of disodium phosphate/potassium phosphate monobasic 0.06 mol/L, pH 8.0) corrected to the appropriate μ by addition of NaCl.
Rutin was first dissolved in a moderate alkaline medium (NaOH, pH <8.5) at about 600 mg/L (over saturated), the undissolved rutin was removed by filtration (LIDA 0.45 μm porosity filters) and then the desired concentrations were obtained by dilution in the appropriate buffer. The concentration of the starting solution was determined by the Folin–Ciocalteau reaction (19) utilizing a calibration curve constructed with rutin dissolved in borate buffer (20) at pH 8.0 which allows complete solubilization of rutin. Permeate concentrations were obtained spectrophotometrically by measuring the absorbance at 370 nm (pH 8.0) and 351 nm (pH 5.0) through calibration curves.

Table 1 Variables tested by the YATES algorithm for selection of the most relevant factors on binding of rutin to bovine serum albumin

	Low level (−1)	High level (+1)
Var. 1 = Ligand concentration (mol/L)	$1.50\cdot10^{-5}$	$1.35\cdot10^{-4}$
Var. 2 = pH	5.0	8.0
Var. 3 = Contact time (h)	3	24
Var. 4 = Ionic strength (mol/L)	0.2	1
Var. 5 = Temperature (°C)	25	55

Table 2 Design matrix of a fractional factorial design with five variables and eight experiments

Experiment	Var. 1	Var. 2	Var. 3	Var. 4	Var. 5
1	−	−	−	+	+
2	+	−	−	−	−
3	−	+	−	−	+
4	+	+	−	+	−
5	−	−	+	+	−
6	+	−	+	−	+
7	−	+	+	−	−
8	+	+	+	+	+

After mixing protein and ligand solutions, the BSA concentration was $4.5\cdot10^{-5}$ mol/L.
Past the desired equilibration time, 2 mL of each mixture were put in a Centrifree unit pre-treated with a blank containing only buffer and ligand at the same sample concentration. The blank samples were also ultrafiltered.
The concentration of the permeate of the blank samples was assumed as total ligand concentration; sample permeate concentration was assumed as free ligand concentration; bound ligand was calculated by difference.
The relative importance of each variable in determining the response y was calculated using the Yates algorithm (21, 22) with the variable levels ordered according to the design matrix shown in **Table 2**.
The value used as response (dependent variable) for each experiment was the percentage of the bound ligand to the total ligand (moles).
Results showed that the variables with the most influence are pH and contact time (data not shown).

Multivariate approach: response surface methodology

Data were collected according to a fractional factorial design. This, if on one side permits the most important causal variables on the response to be evaluated, taking into account each independent variable as well as the interaction between them, and shows how varying them to increase the response itself, on the other side is insufficient to determine the optimal conditions of the reaction.
Performing such task implies to study a response surface that can be mathematically represented by a second degree polynomial equation, which for two causal variables, such as this case, has the following form:

$$y = b_0 + b_1x_1 + b_2x_2 + b_{11}x_1^2 + b_{22}x_2^2 + b_{12}x_1x_2 \quad \text{Eqn [8]}$$

Data collection was designed with the aim to explore adequately the experimental domain, providing the maximum possible amount of information about the problem under investigation, using a minimum number of experiments. An extensive treatment of this subject is given by Clementi *et al.* (21), Box *et al.* (22) and Clementi *et al.* (23).
The central composite design (CCD) provides a suitable way to obtain this set of data. The minimum number of experiments required by the CCD strategy is given

Table 3 Reaction conditions selected for response surface methodology experiments on binding of rutin to Bovine Serum Albumin (BSA) and RubisCO from spinach, according to the central composite design

	BSA		RubisCO	
Code	pH	Time	pH	Time
PP	7.56	13 h 15 min	7.70	13 h 15 min
MM	5.44	4 h 45 min	6.30	4 h 45 min
PM	7.56	4 h 45 min	7.70	4 h 45 min
MP	5.44	13 h 15 min	6.30	13 h 15 min
OL	6.50	3 h	7.00	3 h
LO	5.00	9 h 20 min	6.00	9 h 20 min
OH	6.50	15 h	7.00	15 h
HO	8.00	9 h 20 min	8.00	9 h 20 min
OO	6.50	9 h 20 min	7.00	9 h 20 min
OO	6.50	9 h 20 min	7.00	9 h 20 min
OO	6.50	9 h 20 min	7.00	9 h 20 min
OO	6.50	9 h 20 min	7.00	9 h 20 min

by the formula $2^n + 2n + 4$, where n has the meaning mentioned before.

For two causal variables, pH and contact time, the number of trials is 12, so to the four experiments of an FFD with two variables, that form a square in the causal variables plane, it is sufficient to add a pair of experiments along the co-ordinate axes at the opposite sides with respect to the centre of the design at a distance equal to the semidiagonal of the square, plus four repeated experiments on the centre itself.

The reaction conditions to perform the CCD data collection on the systems BSA–rutin and RubisCO from spinach–rutin are given in **Table 3**.

To carry out the optimization by means of response surfaces two chemometric packages were used: SIMCA - B v. 4.01, developed by Wold *et al.* (24) and the CARSO v. 1.0 procedure, developed by Clementi *et al.* (23).

The sample preparation procedure used for response surface methodology experiments was the same as described for preliminary experiments (Yates algorithm); RubisCO from spinach, which was frozen with glycerol, was dialysed in the needed amounts against selected buffers, in order to obtain the pH values fixed in the central composite design; phosphate buffers at different concentrations (50, 70, 80 mmol/L for pH around 8.0, 6.5 and 5.0, respectively) were used for all samples; the ionic strength was maintained to 0.2 mol/L with NaCl.

Binding studies on RubisCO from tobacco and chlorogenic acid: binding isotherms

Direct partition method. Experiments were carried out in 50 mmol/L, pH 7.3 Tris-HCl buffer, containing NaCl so as to obtain μ = 0.1 mol/L. The protein solution was prepared by separating from the storage buffer by centrifugation an appropriate amount of RubisCO crystals obtained from tobacco and dissolving them in the trial buffer. The protein solution was filtered through LIDA SPA-5003 filters (0.22 µm porosity) and the protein concentration was measured by determining the absorbance at 280 nm, using the coefficient 0.7 to express the RubisCO concentration as mg/mL (25). The O.D. 280 nm / O.D. 260 nm ratio ranged from 1.95 to 1.98.

Two trials were performed: one using the RubisCO protein stored at 4 °C and slowly brought to room temperature (23 °C) and the other with RubisCO reactivated (26) by heating at 50 °C for 20 min and then cooled to room temperature.

The protein concentration in all samples was 18.33 mg/mL, i.e. $3.39 \cdot 10^{-5}$ mol/L. **Table 4** shows the total ligand concentrations and the ratios between the concentrations of the ligand and the protein for both experiments. Molecular weights of 354.3 and 540,000 Da were assumed for chlorogenic acid and RubisCO, respectively.

Each sample was prepared in duplicate, with a blank solution containing buffer instead of the protein solution. Separation of free ligand from the sample mixtures was done using presaturated Centrifree units with a 10,000 cut-off filled with 0.3 mL of solution and centrifuged at 4000 rpm for 7 min. The chlorogenic acid concentration was determined by measuring the absorbance at 325 nm making use of a calibration curve; highly concentrated samples were diluted before reading and at least duplicate dilutions were done when the two replicated readings were different. Data plots and regression lines were drawn taking into account all data collected for each sample.

Raw data were treated as follow: the permeate concentration of chlorogenic acid represented the free ligand $[Lf]$, the permeate concentration of the blank samples represented the total ligand and the difference between the blank and $[Lf]$ was assumed as the concentration of the bound ligand; the ratio between bound ligand and protein concentrations is the molar binding ratio (r), while the ratio between r and $[Lf]$ is the parameter

Table 4 Ligand concentrations and ligand to protein molar ratios used in the binding experiment carried out on RubisCO from tobacco and chlorogenic acid by the direct partition ultrafiltration method. Reaction conditions: Tris-HCl buffer 50 mmol/L, pH = 7.3, μ = 0.1 mol/L; protein concentration = 18.33 mg/ml

Untreated RubisCO		Heated RubisCO	
$[Lf] \cdot 10^5$	$[L]/[P]$	$[Lf] \cdot 10^5$	$[L]/[P]$
2.995	0.88	2.795	0.78
7.740	2.26	7.943	2.21
15.966	4.67	16.028	4.47
25.418	7.42	26.544	7.40
44.966	13.13	36.598	10.20
64.082	18.72	46.087	12.85
97.754	28.55	65.684	18.31
130.024	37.98	81.296	22.67
		100.693	28.07
		118.381	33.00
		134.668	37.54
		165.657	46.18

used as the dependent variable in drawing the Scatchard plot (27, 28).

Diafiltration method. The experimental conditions during the trial were: RubisCO concentration at 9.688 mg/mL (0.0179 mmol/L); Stock chlorogenic acid concentration at 28.95 mg/L (0.0817 mmol/L); Buffer: phosphate buffer 50 mmol/L, pH 7.0, μ = 0.3 mol/L; Initial flow rate at 0.4 mL/min; Average fraction volume at 2.96 mL; Initial cell volume at 33 mL; Average cell volume was 28.9 mL; Total volume of permeate collected was 106 mL.

Raw data were treated as above described Eqns 1 to 7, following the procedure described by Cheryan and Saeed (29). **Figures 3, 4** and **5** show three typical utilizations of these data: the experimental and theoretical dilution curves, the binding isotherm and the Scatchard plot, respectively.

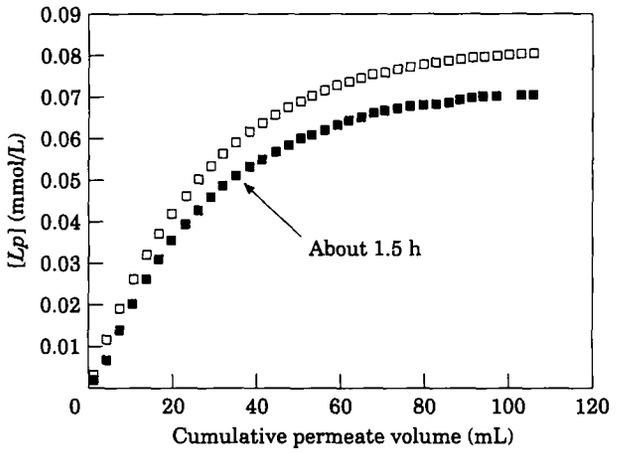

Fig. 3 Ideal (□) and experimental (■) dilution curves obtained from the diafiltration experiment carried out on RubisCO from tobacco and chlorogenic acid. Reaction conditions: RubisCO concentration = 0.0179 mmol/L; stock chlorogenic acid concentration = 0.0817 mol/L; buffer = phosphate buffer 50 mmol/L, pH 7.0, μ = 0.3 mol/L; initial flow rate: 0.4 mL/min; medium fraction volume: 2.96 mL; medium cell volume: 28.9 mL. [*Lp*] = permeate ligand concentration.

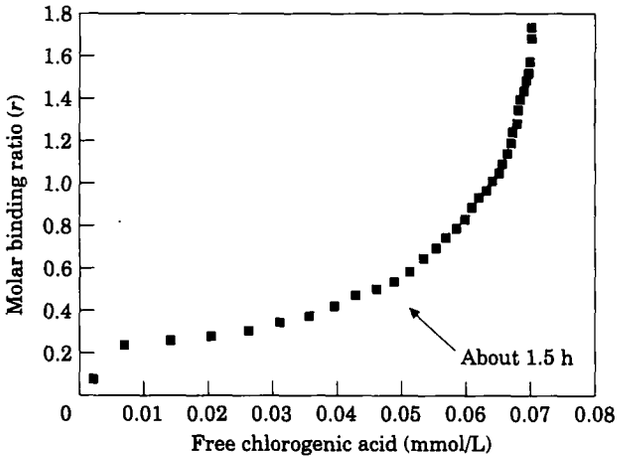

Fig. 4 Binding isotherm obtained from the diafiltration experiment carried out on RubisCO from tobacco and chlorogenic acid. The reaction conditions are listed in **Fig. 3**

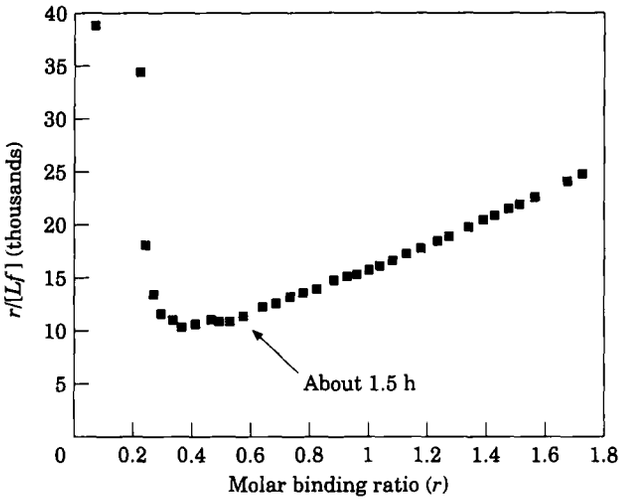

Fig. 5 Scatchard plot obtained from the diafiltration experiment carried out on RubisCO from tobacco and chlorogenic acid. The reaction conditions are listed in **Fig. 3** [*Lf*] = [*Lp*] as defined in **Fig. 3**

At the end of the diafiltration run, the cell content was filtered and the absorbance at 325 nm was measured; the protein concentration of the filtrate was measured by the Bradford method (30,31) and expressed as mg/mL of RubisCO by comparison with a calibration curve.

Results and Discussion

Multivariate approach: response surface methodology

By a direct comparison of data regarding *r* values (data not shown), collected on both the systems BSA–rutin and RubisCO from spinach–rutin according to a CCD, RubisCO showed a greater affinity towards the ligand rutin with respect to BSA; moreover, for both proteins saturation of the binding sites was not reached.

The optimization study was performed using the CARSO procedure that implies a nonlinear partial least squares regression (PLS), using as causal variables pH (x_1) and contact time (x_2), and the value of *r* as response.

For the BSA system both the variables in their linear terms and the bifactorial cross products were the most relevant in the modelling of the response surface (**Table 5**).

The canonical analysis performed on the polynomial equation determined, for searching for the co-ordinates of the stationary point, together with the eigenvalues, showed that the stationary point is a saddle point or a minimax whose co-ordinates are out of the experimental domain, being the value of the pH 4.78, identical with the isoelectric point of the protein, and the corresponding time value, negative. The projection of the response surface on a plane (isoresponse plot) is shown in **Fig. 6a**.

Table 5 Loadings of the causal variables obtained with the PLS regression on the BSA–rutin and RubisCO from spinach-rutin systems

	BSA–rutin			RubisCO from spinach–rutin		
var.	beta1	beta2	beta3	beta1	beta2	beta3
x_1	0.87	0.11	0.40	0.88	0.18	–0.20
x_2	0.43	0.18	–0.82	0.33	0.07	–0.07
x_1^2	–0.10	–0.10	–0.11	–0.27	0.42	–0.92
x_2^2	–0.01	–0.05	–0.39	0.14	–0.89	–0.32
x_1x_2	0.23	–0.97	–0.05	0.14	0.03	–0.03

x_1 = pH; x_2 = contact time.
beta1 = loadings of the first latent variable; beta2 = loadings of the second latent variable; beta3 = loadings of the third latent variable.

Table 6 Lagrange analysis on the BSA–rutin and RubisCO from spinach–rutin systems

BSA–rutin		RubisCO from spinach–rutin	
pH; time	*r*	pH; time	*r*
pH5; 3 h (–1; –1)	0.179	pH6; 3 h (–1; –1)	0.198
pH5; 15 h (–1; +1)	0.166	pH6; 15 h (–1; +1)	0.213
pH8; 3 h (+1; –1)	0.338	pH8; 3 h (+1; –1)	0.368
pH8; 15 h (+1; +1)	0.687	pH8; 15 h (+1; +1)	0.534

Values in parentheses represent the coded values of the causal variables.

The Lagrange analysis, used for searching for values internal to the experimental domain, gave the results shown in **Table 6**. Analysing them, it appears that the time of contact between the two reagents is insignificant in determining the response at low pH, while it is important at higher pH values; an explanation of this may be referred to the rutin autooxidation, stronger in alkaline solutions.

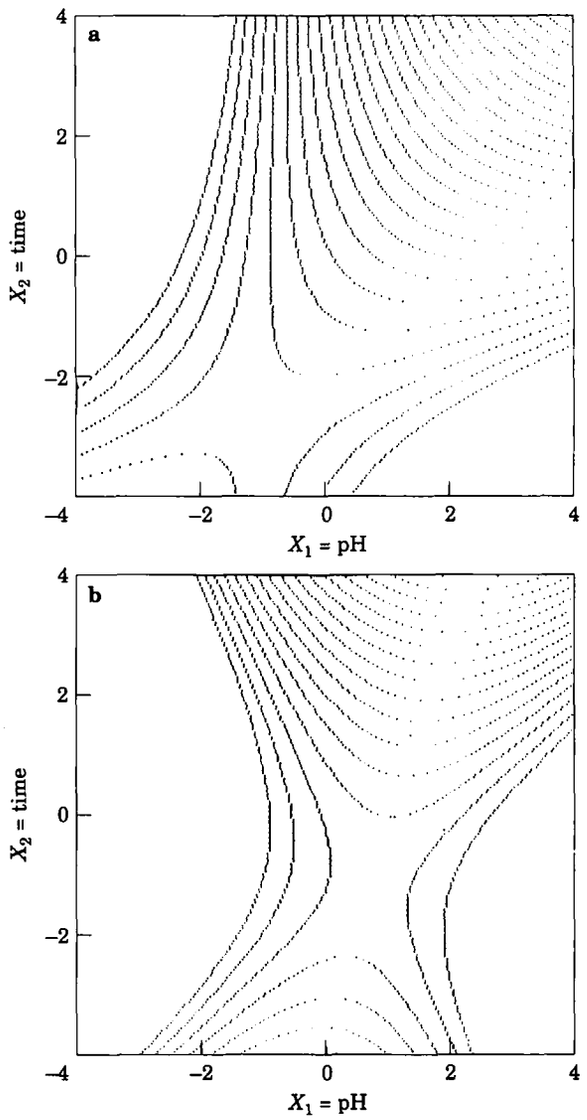

Fig. 6 Isoresponse plots of the optimization of the interaction of the two systems BSA–rutin (**a**) and RubisCO from spinach–rutin (**b**)

For RubisCO from spinach the most important variables in describing the latent variables are pH in its linear term and time in its quadratic term. For this protein a response surface with a saddle point was obtained too, but now the co-ordinates of such a point are internal to the experimental domain: pH 7.67 and time of contact 1 h 55' with an *r* value of 0.37 (**Fig. 6b**). Observing the data obtained with the Lagrange analysis one can see that the effect of time in slightly acid solutions is negligible, while it becomes important at higher pH values (**Table 5**).

The results so far obtained give no information about a correlation between the RubisCO affinity towards rutin on the one hand and the modification of the protein charge distribution on the other; differently from BSA a saddle point at a pH value corresponding to the isoelectric point was not found. A possible reason for such behaviour could depend on the high charge to mass ratio that enables interaction between RubisCO with the ligand even when the number of the negative charges is identical to the positive ones.

Furthermore the influence of pH on binding may be related to rutin autooxidation rather than to a conformational change of the protein.

Classical binding study by the direct partition technique

Data obtained from direct partition experiments were used to draw binding isotherms, semilogarithmic plots, double reciprocal plots and Scatchard plots. According to Connors (15) it is useful to compare information derived from different methods of data treatment.

A double reciprocal plot is a plot of $1/r$ *vs.* $1/[Lf]$. The first goal is to determine n, the maximum number of binding sites on the macromolecule: this can be obtained from the intercept on the y-axis of the double reciprocal curve as $1/[Lf]$ tends to zero, whose intercept on the y-axis yields the parameter $1/n$. The values obtained from the two experiments were: 0.187 (with a standard error of 0.054) for the reactivated RubisCO and 0.198 (with a standard error of 0.062) for the

untreated RubisCO, i.e. values of n of 5.3 and 5, respectively. The double reciprocal plot also yields the x-axis intercept, whose meaning, when nonidentical sites are present, represents a combination of the different association constants (K_a) with the number of sites in each class of sites, e.g. considering two classes of sites with two different K_a, the x-axis intercept is: $(\Sigma n)\cdot K_1\cdot K_2 \,/\, (n_1\cdot K_2 + n_2\cdot K_1)$.

Scatchard plots of the two experiments are shown in **Fig. 7**. In both cases an upward curved plot was obtained, which may be the result of either negative cooperativity or site heterogeneity. In order to approach a stoichiometry hypothesis, some initial estimates are to be done: it is possible to assume, basing on Klotz and Hunston considerations (32), that the initial slope of the Scatchard plot roughly approximates the first association constant, K_1, regardless of the relative magnitude of the others; the intercept of the y-axis represents the overall association constant, i.e. $\Sigma n_i\cdot K_i$ where i is a generic class of sites. The values obtained are shown in **Table 7**.

Considering the hypothesis of nonidentical classes of sites, the data suggest that the first association constant, the highest one, was minimally modified by the heat treatment, however an additional, low affinity, binding component was markedly reduced, causing a reduction of the overall binding constant of 0.225. Several attempts were made, in order to figure out a combination of K_1, n_1, K_2 and n_2, with $n_1 + n_2 = 5$ that would adequately fit into the formula of the x-axis intercept of the double reciprocal plot described by Klotz and Hunston (32) as mentioned above, giving the two values experimentally found (–1545 for reactivated RubisCO and –2600 for untreated RubisCO); at the same time different combinations of the above parameters were tried which would give satisfying values for the initial slopes in the Scatchard plot and the x-axis intercepts in the double reciprocal plot in both experiments. Obviously it can be difficult to draw conclusions from experimental data, when a high number of sites and more than one association constant are present.

We can suggest a model with one, high affinity site of nonhydrophobic nature together with four hydrophobic sites. A fairly good fitting for the parameters above described was obtained with the values presented in **Table 8**.

In the heat reactivated RubisCO, the decrease of the overall binding constant without a noticeable reduction in K_1 might be explained by the conformational change associated with reactivation, according to Kawashima *et al.* (26) and Chollet and Anderson (33); in fact the hexadecameric structure of RubisCO would increase in compactness, with a reduced exposure of the hydrophobic areas: in this way a lower affinity to the hydrophobic sites would be shown.

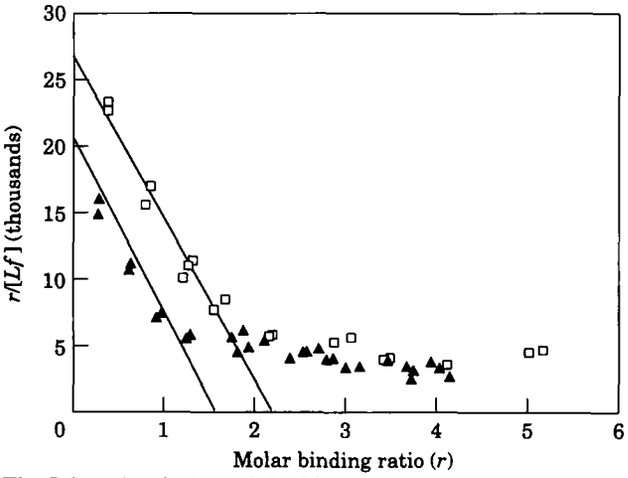

Fig. 7 Scatchard plots of the binding experiments carried out on RubisCO from tobacco and chlorogenic acid by the direct partition ultrafiltration technique. Plots were drawn with all data obtained for each experimental point. Solid lines represent regression lines obtained for the first eight experimental points in both experiments (□ = untreated RubisCO; ▲ = treated RubisCO). Standard errors of y intercept estimation = 1366 □ and 2273▲; Standard errors of slope = 1030 □ and 2041 ▲

Table 7 Values of y-axis intercept, slope and correlation coefficient for untreated and reactivated RubisCO

	Untreated RubisCO	Reactivated RubisCO
y-axis intercept	26835	20794
Slope	–12213	–13345
Correlation coefficient	0.95	0.81

Classical binding study by the diafiltration technique

As far as obtaining of the binding parameter is concerned, the diafiltration experiment showed a definitely atypical behaviour: in fact the Scatchard plot does not look similar to the negative or to the positive cooperativity case (**Fig. 5**). After the first seven fractions the affinity of the ligand to the protein seems to increase sharply, until the point that small increments in the ligand concentration (at the end of the run) result

Table 8 Binding parameters obtained from binding experiments carried out on tobacco RubisCO and chlorogenic acid by the direct partition ultrafiltration method. The reaction conditions are listed in **Table 3**. Starting parameters obtained by regression procedures from experimental data are labelled with *; other data were obtained by a trial and error fitting procedure

	Untreated RubisCO	Heated RubisCO
K_1	17500	16500
n_1	1	1
K_2	2334	1073
n_2	4	4
n_{tot}	5*	5*
Initial slopes of the Scatchard plot	–12214*; –12224	–13345*; –13314
y-axis intercept of the Scatchard plot	26835*	20794*
x-axis intercepts of the Double reciprocal plot	–2600*; –2823	–1545*; –1320

in increasing increments of bound ligand. One and one-half to two hours after the beginning of the run, increasing milkiness started to appear in the cell solution; this is due to the mechanical instability of RubisCO, which should not however take place at such protein concentrations (about 10 mg/mL) (34, 35). A separate mechanical stability experiment (data not shown) revealed an average concentration decrease of 0.0375 after 2 h under stirring; the analysis of cell protein concentration (see Materials and Methods section) showed an even smaller (not analytically significant) decrease. However, a mass balance around the cell was calculated, which demonstrates that of 3.05 μmol of ligand present in the cell at the end of the run, 2.026 μmol were free ligand (assuming the same ligand concentration as the last permeate fraction) and 1.023 μmol should be divided between membrane, soluble protein, insolubilized protein; membrane adsorption was estimated as 0.288 μmol using data from previous blank (without protein) runs, while 0.123 μmol were attributed to soluble protein based on the difference in O.D. between filtered cell content and the last permeate fraction; the difference, 0.613 μmol, was consequently bound to the minimum amount of aggregate protein, which would hence reveal a very high affinity to the ligand. It is impossible to determine the exact amount of RubisCO which underwent this phenomenon, but it is reasonably less than 0.0375 (see above). Such an amount would mean 22.2 nmol, i.e. a molar binding ratio of 0.613/0.0222 = 27.6 moles of ligand per mole of protein. These findings should be confirmed by further experiments, however, this result is of great importance from a technological point of view: in fact it might mean that the maximum damage in terms of phenolic contamination does not happen during extraction steps, when the protein is still soluble, but during the precipitation–crystallization step when it aggregates.

Conclusions

In this paper it was seen that it is possible to obtain important information about the binding of phenolic compounds to proteins by means of optimization studies as well as classical methods such as ultrafiltration techniques. The collection of data in accordance with a central composite design and the following optimization with the CARSO procedure has given results indicating that the behaviour of the two proteins, BSA and RubisCO from spinach, towards the rutin is different. Precisely for BSA the interaction reduces going to lower values of pH up to the isoelectric point, while for RubisCO there is no correlation between the decreasing of the interaction and such point. Moreover RubisCO shows a higher affinity to the ligand.

Applying these results to technological processes one may reduce the working time and lower the pH value.

Among the ultrafiltration techniques, the direct partition one is more advisable for its speed, important in systems easily degradable, and the possibility of working with small amount of substances. On the other hand diafiltration permits determination of a high number of experimental points in a short time, but its reliability is high when dealing with systems that show an affinity of the protein towards the ligand higher than the one tested in this paper; this allows a better definition of the initial portion of the Scatchard plot, because saturation of binding sites on the protein can be reached without using too high a concentration of the ligand in the reservoir.

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