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Conglutin γ, a lupin seed protein, binds insulin in vitro and reduces plasma glucose levels of hyperglycemic rats

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Abstract

This work describes the in vitro interaction between a lupin seed protein, namely, conglutin γ, and insulin. The binding to an insulin-immobilized matrix occurs in the pH range from 7.5 to 4.2 and is strongly affected by ionic strength, suggesting that it is driven primarily by electrostatic interactions. The quantitative parameters of the binding were determined by surface plasmon resonance. On the basis of the conditions required for the interaction to take place and the quantitative binding parameters, it appeared that the interaction is specific, despite the fact that the origin of the two protein molecules is completely different. The effect of the oral administration of conglutin γ on the glycemic levels of rats subjected to glucose overloading was a statistically significant reduction in glycemia comparable to that of metformin, a well-known glucose lowering drug. These findings represent the first molecular evidence of the possible use of a legume protein in the control of glycemia.

Keywords: Seed protein; Legumes; Insulin; Protein–protein interaction; Surface plasmon resonance; Glycemia

1. Introduction

Storage proteins are the main protein constituents of most seeds, and legume seed proteins represent the major dietary plant protein source. Legume seeds also contain a number of biologically active proteins that play many specialized roles. Of these proteins, the lectins, hydrolase inhibitors, ribosome inactivating proteins (RIPs), and allergens are traditionally considered as antinutritional compounds [1]. However, some could favorably be reconsidered in view of the potential exploitation of their biological behavior in pharmacological, medical, cosmetic, and food applications. In this respect, the search for novel applications is currently expanding.

Although direct demonstrations of the capacity of legume seed protein to control metabolic disorders are still scarce, some studies are currently emerging. For example, the cholesterol lowering effect of soybean 7S globulin α′ subunit in cell [2] and animal [3] model systems and the anticarcinogenic activity of legume Bowman-Birk trypsin-chymotrypsin inhibitors [4] have been reported. Therefore the possibility that specific legume seed proteins, either alone or in combination with other seed components, may exert a biological effect in humans, is no longer a mere hypothesis.

White lupin (*Lupinus albus*, L.) seed is a legume grain with a protein content of about 40% on a dry weight basis [5]. The bulk of lupin proteins belongs to the vast families of 11S and 7S globulins [6]. In this latter group one protein, named conglutin γ [7], accounts for about 5% of the total globulins [6]. Conglutin γ is a glycoprotein of M_r relative molecular mass around 47 kDa. It is composed of two disulphide bridged subunits of 29 and 17 kDa [8]. Conglutin γ displays unique properties of its own, since its amino acid sequence does not match any other legume protein canonical sequence [9]: it binds divalent metal ions, especially Zn^{++} and Ni^{++} [10], it is not cleaved during seed germination [11] and it is also unusually resistant to in vitro proteolysis, unless fully denatured [12]. Proteins homolo-

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gous to conglutin γ, for which the biological function is still to be unequivocally assessed, have also been found in other plant species, including soybean [13] and carrot [14].

In the present work we show the binding capacity of lupin conglutin γ to insulin and determine some characteristics of this interaction by affinity chromatography and the binding quantitative parameters by surface plasmon resonance. In addition, preliminary animal trials aiming to demonstrate the effect of conglutin γ administration on rat glycemic levels were carried out, and the results are reported.

2. Methods and materials

2.1. General

Dry mature seeds of white lupin (*Lupinus albus* L, var. Multitalia) were kindly provided by Dr. Massimo Fagnano, University of Naples, Italy. Chemicals were all reagent grade from Sigma-Aldrich (St. Louis, MO) and Bio-Rad (Hercules, CA). Insulin-FITC (catalog no. I2383), used for SPR experiments, and insulin coupled with agarose beads (catalog no. I2508) were also obtained from Sigma-Aldrich.

2.2. Purification of conglutin γ

Conglutin γ was purified as described previously by using a combination of anion and cation exchange chromatography [15]. The purified protein was freeze-dried and resuspended in the appropriate buffers before use.

For the estimation of conglutin γ concentrations, optical measurements at 280 nm were made. The extinction coefficient of 1 for a solution of 1 mg/mL was used [16].

2.3. Immobilized insulin affinity chromatography

Conglutin γ solutions were loaded onto an insulin-agarose column equilibrated in 25 mmol/L Tris-HCl, pH 7.2, and eluted by addition of 0.2 mol/L NaCl to the loading buffer.

For the pH-dependent binding assays, the following buffers at the desired pH values were used: 25 mmol/L Tris-HCl, 25 mmol/L 2-morpholinoethanesulfonic acid, and 25 mmol/L Na-acetate.

For the experiments involving protein denaturation, conglutin γ was treated with 8 mol/L urea in the affinity chromatography loading buffer. The denatured protein solution was then diluted to 2 mol/L urea with the same buffer, before loading onto the insulin affinity column.

2.4. Determination of quantitative binding parameters by surface plasmon resonance

A BIACORE X system (Uppsala, Sweden) was used to analyze molecular interactions by means of surface plasmon resonance (SPR) [17]. Conglutin γ was covalently linked to a carboxymethylated dextran surface, CM5 sensor chip, by using amine-coupling chemistry [18]. A surface density of 10,800 resonance units (RU) was generated for conglutin γ by using 70 μL of a 10 μg/mL protein in 10 mmol/L acetate buffer, pH 4.5. Solutions of the interacting protein, namely, the analyte insulin, were injected over the surface at 25°C with a flow rate of 3 μL/min in running buffer (10 mmol/L HEPES, pH 7.4, containing 0.005% (v/v) Tween 20). After injection, analyte solutions were replaced by running buffer at a continuous flow rate of 3 μL/min. Surface regeneration was accomplished by injecting 0.5 mol/L NaCl (3 μL/min; 1-minute contact time). All analyte solutions were run simultaneously over a control flow cell containing a blank surface (with no immobilized protein) at 25°C. Each sensorgram (time course of the SPR signal) was subtracted for the response observed in the control flow cell and normalized to a baseline of 0 RU. Different concentrations of the analyte were passed for 10 minutes over the sensor chip with immobilized conglutin γ (30 μL injections at a flow rate of 10 μL/min). The interaction rate constants were calculated by using the BIA evaluation 3.1 SPR kinetic software (BIAcore).

2.5. Electrophoretic techniques

SDS-PAGE was carried out on 12% polyacrylamide gels, according to Laemmli [19] under nonreducing conditions using a mini-Protean II cell (Bio-Rad). The gels were stained with Coomassie Blue.

2.6. Animal studies

A total of 100 male rats (Charles River, Calco, LC, Italy) with an average body weight of 275–300 g were maintained under stable conditions for 7 days before the experiment. The animals were given a standard rat diet and were kept under automatically controlled light, temperature, and humidity conditions. The rats were divided into five groups. One group, the control group, received only the vehicle (1% carboxymethylcellulose, CMC); another three groups received conglutin γ at concentrations of 30, 60, or 120 mg/kg body weight in 1% CMC; and the last group was given 50 mg/kg body weight of metformin in 1% CMC. Administration was carried out by gavage 30 minutes before the glucose overloading experiment. At time 0 of the experiment, each rat was given 2 g/kg body weight glucose administered orally. At established times thereafter (30, 60, 90, and 120 minutes from glucose administration), each rat was treated with 50 mg/kg body weight Na-thiopenthal, and 5 mL of blood was collected in 7.5-mmol/L EDTA containing tubes. The blood was immediately centrifuged at $2000 \times g$ at 4°C for 10 minutes and the supernatant used for glucose assays.

All procedures involving rats and their care were performed according to the Italian Government Guidelines for animal tests and were in agreement with the European Commission rules (86/609/EEC).

2.7. Plasma glucose assays

Plasma glucose concentrations were determined by the enzymatic method using the Glucose-Trinder kit from Sigma-Aldrich (catalog no. 315-500) and were performed

in triplicate for each plasma sample. Data are expressed as grams per liter and reported as mean±SE.

2.8. Statistical analysis of animal trials

Statistical analyses of the differences of plasma glucose concentrations in each rat group versus the control (i.e., the vehicle alone) were evaluated with the Dunnet test [20]. Differences were considered significant at the level of $P < 0.05$.

3. Results

3.1. Insulin binding studies by affinity chromatography

The elution pattern of conglutin γ from the immobilized-insulin chromatographic resin is shown in Fig. 1, panel A. Loading of conglutin γ on the column, at the protein and salt concentrations and the pH detailed in the Methods and materials section, led to the complete retention of the protein. Desorption was achieved by the addition of 0.2 mol/L NaCl to the elution buffer. When the same experiment was performed with a linear gradient of NaCl concentration, elution of conglutin γ was seen to begin at 40 mmol/L ionic strength and to peak at 85 mmol/L (data not shown). Therefore, the desorption of conglutin γ from insulin-bound matrix occurred over a relatively small range of ionic strength, suggesting that the binding is primarily driven by electrostatic forces (discussed below). To exclude the possibility of either a precipitation of the protein in the column or a nonspecific binding to the resin at low ionic strengths, a Sepharose CL-2B column (Sigma-Aldrich, St. Louis, MO), an agarose-based matrix, was used under the elution conditions detailed above: conglutin γ was not retained by this resin (data not shown). The inset of the figure shows the SDS-PAGE pattern of the protein eluted from the column (Fig. 1, inset, lane 1). Complete binding of conglutin γ to the column was also observed when a lupin total protein extract was loaded on the column (Fig. 1, inset, lane 2: unbound material; lane 3: bound conglutin γ). This indicates that the presence of other proteins and minor compounds did not prevent the occurrence of the interaction, which therefore appeared highly specific. Chromatographic experiments in the presence or absence of 10 μmol/L Zn^{++} or 10 mmol/L EDTA were performed in relation to the divalent metal ions binding capacity of conglutin γ [10]. In both cases, the binding was not affected (not shown). The preservation of conglutin γ native conformation appears to be a prerequisite for the binding to occur. Indeed, when 8 mol/L urea pre-treated conglutin γ was loaded on the insulin immobilized column under conditions allowing the interaction to take place, i.e., 2 mol/L urea (Fig. 1, panel B, continuous line), no binding took place (Fig. 1, panel B, broken line).

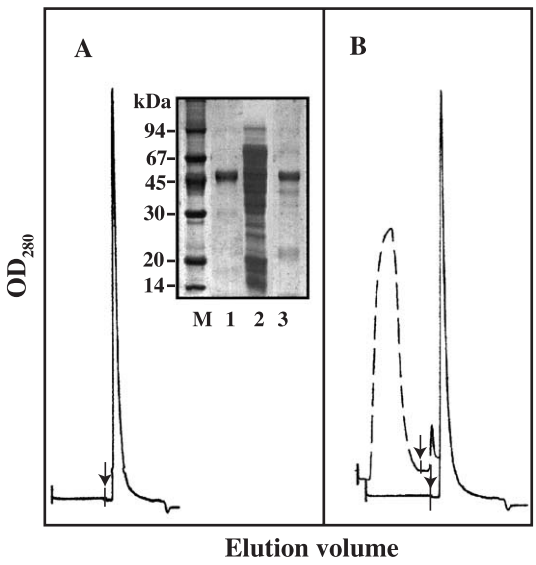

Fig. 1. Insulin-affinity chromatography profiles of lupin conglutin γ. (A) Native purified conglutin γ (1 mg) was loaded on an insulin-agarose column in 25 mmol/L Tris-HCl buffer, pH 7.2. The arrow indicates the application of 0.2 mol/L NaCl to the elution buffer. In the *inset*, the SDS-PAGE pattern under nonreducing conditions shows a band of M_r around 50 kDa, which is characteristic of the unreduced conglutin γ polypeptide. Total lupin globulin extract (10 mg) was also loaded onto the column (data not shown). *Lane 2* shows the pattern of the unbound fraction; *lane 3* shows that of the bound fraction. M = standard reference proteins. (B) Conglutin γ was treated with 2 mol/L urea in the loading buffer. After this treatment, the elution profile obtained is represented by the continuous line. The broken line shows the elution profile of conglutin γ pre-treated with 8 mol/L urea in the loading buffer, then brought to 2 mol/L urea immediately before loading to the column. As in panel A, the arrows indicate the application of 0.2 mol/L NaCl to the elution buffer. Experimental details are given in the Methods and materials section.

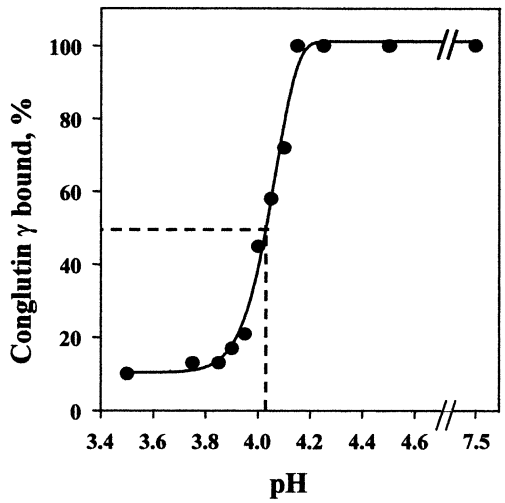

Fig. 2. The pH dependence of the binding of conglutin γ to insulin affinity chromatography resin. The diagram shows the residual amounts of conglutin γ bound to the resin, as a percentage of the total applied amount (1 mg), at the different pH values tested. Experimental details are given in the Methods and materials section.

The dependence of conglutin γ binding to insulin on the pH is shown in Fig. 2. The same insulin affinity column as in the previous experiment was used. Binding was complete at any pH value from 7.5 to 4.2. On the other hand, a very small pH drop below that value caused the almost complete release

of the protein, with a half binding value close to pH 4.0. Interestingly, this pH represents the pK_a mean value between glutamic and aspartic acid side groups, thus supporting the conclusion regarding the role of the electrostatic interactions on the binding of conglutin γ to insulin.

3.2. Determination of quantitative binding parameters by SPR

SPR was used to describe the real time association and dissociation of conglutin γ to insulin. Conglutin γ was therefore coupled directly to the sensor chip. The binding and release of insulin to and from this chip was followed. The kinetics of binding were reproducible in the different experiments. Fig. 3 depicts a typical sensorgram obtained by using insulin concentrations in the range from 10 to 800 μmol/L. The Scatchard plot, obtained using the maximum RU for each given insulin concentration, is shown in the inset. A K_d of 9.2×10^{-5} mol/L was calculated from the slope of the plot. Simultaneous fitting of sensorgrams with BIA evaluation software allowed the estimation of the association and dissociation rate constants, k_{on} and k_{off} for the binding to insulin, which were 2.7 M^{-1} s^{-1} and 2.0 s^{-1}, respectively, yielding a calculated K_d of 7.2×10^{-5} M. The similarity of the K_d values, obtained from the two different methods [21], supports the validity of the interpretation of the data.

3.3. In vivo plasma glucose reduction by oral administration of conglutin γ to hyperglycemic rats

In the experimental conditions used (detailed in the Methods and materials section), the effect of the oral administration of conglutin γ to rats under glucose overloading produced a significant reduction of plasma glucose with respect to the control (Fig. 4). This effect was greatest

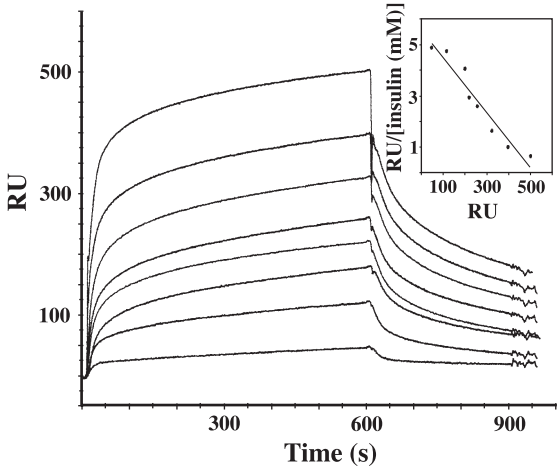

Fig. 3. Association/dissociation kinetics for the binding between conglutin γ and insulin. Conglutin γ was immobilized on the sensor chip and different concentrations of insulin (10, 25, 50, 75, 100, 200, 400, and 800 μmol/L, from the bottom to the top of the graph) were flowed onto the chip surface. The maximal binding value obtained for each insulin concentration was used to generate the Scatchard plot reported in the *inset.*

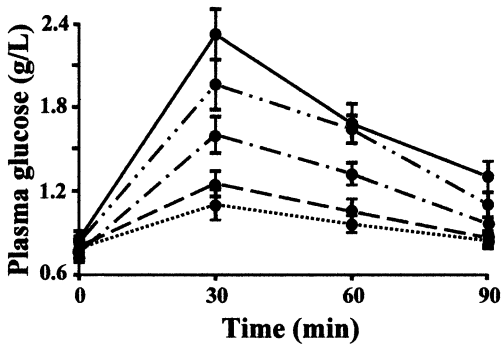

Fig. 4. Influence of conglutin γ on plasma glucose concentrations of rats during glucose overloading trials. Plasma glucose was assayed in rats at various times from glucose overload (control: continuous line) upon pretreatment with 30 ("dash-dot-dot" line), 60 ("dash-dot" line), 120 (broken line) mg/kg body weight of conglutin γ or 50 mg/kg body weight metformin (dotted line). Each value represents the mean of five rats. Vertical bars indicate standard error of each mean value.

at 30 minutes from the glucose overloading and persisted until 60 minutes. After 90 minutes, glucose levels were not statistically different from the control group. The observed effect appeared to be dose-dependent: in particular, the effect of the highest conglutin γ dose did not differ statistically from that measured with metformin, a well-known glucose lowering drug [22].

4. Discussion

This work describes the interaction in vitro between a lupin seed protein, namely, conglutin γ, with insulin. The results obtained with insulin affinity chromatography suggest that the binding is primarily driven by electrostatic forces, as is the case when there is interaction between insulin and one monomer of the insulin receptor [23]. The native structure of conglutin γ is a prerequisite for the binding, indicating that the interaction requires a specific conformation of the protein. In addition, although the quantitative binding parameters show a K_d value for the interaction as great as 10^{-5} M, it appeared that the interaction is highly specific, as shown by the selectivity of the affinity chromatography separation of a lupin total protein extract. This finding is even more interesting if one considers that the two protein molecules are of totally different origin.

As far as the in vivo assays is concerned, a reduction of the glycemic levels in hyperglycemic rats was induced by oral treatment with conglutin γ. The lowering effect of conglutin γ at the highest dose was comparable to that obtained with approximately a half dose of metformin with respect to conglutin γ. The present results do not as yet allow any hypothesis to be drawn regarding the mechanism by which conglutin γ exerted the observed effect. Nevertheless, the plasma glucose lowering effect described in this work may represent a rationale behind the use of lupin seed decoctions as an antidiabetic drug, as described in the old pharmacopoeia [24]. After the description of the glycemic

lowering activity of "a lupin-derived yellowish powder containing a putative insulin-like active principle, which persisted during seed germination" in 1940 [25], no further attempts to identify the molecule responsible for this effect were made. The present findings, by showing a specific interaction of conglutin γ with insulin and the effects on the animal models, candidate this protein as the molecule responsible for the regulatory effect of lupin seeds on glycemia. Further studies at both the molecular and pharmacological level, aimed at understanding the possible mechanism(s), and assessing any potential exploitation—especially in the expanding area of pre-clinical diabetes treatment—are currently being undertaken.

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