Heat-Induced Aggregation of *Phaseolus vulgaris* L. Proteins: An Electron Spin Resonance Study

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The mechanism of heat-induced aggregation of *Phaseolus vulgaris* L. proteins and of subunit interactions of importance for susceptibility of proteins to proteolysis was studied by electron spin resonance (ESR) spectroscopy. The mobility of a spin label bound to lysine residues was monitored at two different pH-induced (neutral and alkaline) association states of proteins extracted from raw and cooked common bean. The molecular weight of the protein complexes was assessed by size exclusion-high performance liquid chromatography (SE-HPLC) of labeled proteins. Upon alkaline dissociation, both native and denatured protein subunits underwent a reassociation process to form soluble complexes of molecular weight higher than the species originally present at neutral pH. However, unlike native proteins, impaired mobility of the spin label was observed in the aggregates that are formed after dissociation of subunits of denatured proteins, indicating a reduced accessibility of lysine residues. Trapping of lysine residues inside protein aggregates may explain limited digestibility in the small intestine of proteins in cooked legumes.

Keywords: *Legume proteins; aggregation; ESR*

INTRODUCTION

Structural−functional requirements of legume storage proteins are aimed at ensuring efficient packaging of protein into protein bodies of the seed until germination. This is accomplished by a multimeric structure as it is typical of the major storage protein 11*S* (leguminlike) and 7*S* (vicilin-like) globulins of legume seeds. Indeed, in such proteins monomeric subunits are closely held together by hydrophobic forces, electrostatic interactions, hydrogen bonding, and, possibly, disulfide bonds (Derbyshire et al., 1976; Kinsella et al., 1985). These factors are also consistent with low solubility of the reserve protein for efficient deposition into the seed, compact structure, and propensity for self-aggregation (Kinsella et al., 1985; Carbonaro et al., 1993).

Although variation in the ratio of the two globulins exists among legume species (Wright, 1987; Shewry et al., 1995), the significance of the occurrence of two different major storage globulins in seeds has not been completely clarified. However, despite the differences in subunit composition (number and type), molecular weight, and association−dissociation behavior as a function of pH and ionic strength, both globulins undergo dissociation into their constituent subunits upon thermal treatment, with reassociation of monomers and formation of either soluble or insoluble complexes (Derbyshire et al., 1976; Utsumi et al., 1984; Carbonaro et al., 1997). Indeed, a marked loss of solubility has been observed to occur after heating of several legume species, and the mechanism of aggregation of 11*S* versus 7*S* denatured globulin has been examined with purified soy proteins (German et al., 1982; Utsumi et al., 1984).

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We previously studied the solubility behavior in different conditions of pH and ionic strength of proteins extracted from raw and cooked legumes (Carbonaro et al., 1993, 1997). We found that, in addition to hydrophobic forces, electrostatic interactions among subunits play a role in governing the solubility of both native and denatured proteins. The involvement of basic residues in the stabilization of heat-induced protein aggregates was supposed to affect the rate and extent of protein digestion by trypsin in the small intestine. In addition, we recently provided evidence that insolubilization of legume proteins induced by heating has a detrimental effect on their digestibility either in vitro or in vivo (Carbonaro et al. 1997, 1998).

In an effort to gain further information about the mechanism of heat-induced association of legume proteins at a molecular level and on the subunit interactions of relevance for digestibility of proteins, we have applied the electron spin resonance (ESR) spin labeling technique to legume proteins (Berliner, 1976). ESR spectroscopy is a suitable magnetic resonance tool for obtaining information about the rotational mobility of nitroxide spin-labeled macromolecules over a wide range of rotational correlation times. It is based upon the magnetic activity of radicals, like nitroxide, that undergo electron spin transitions when excited in the presence of an external magnetic field. The intensity of the nitroxide signal is related to the amount of radical incorporated into the macromolecule. The line shape of the spectrum, on the other hand, reveals the degree of hindrance of the radical in its binding site.

Although ESR spectroscopy has been successfully employed in studying molecular properties of gluten protein systems (Pearce et al., 1987, 1988; Hargreaves et al., 1994) and structural modifications of gelatinized starch (Nolan et al., 1986), to our knowledge this technique has not been used hitherto to study legume

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proteins. In this study, we examined the mobility of a spin label bound to lysine residues at two different pH-induced (6.5 and 12.0) association states of proteins extracted from common bean (*Phaseolus vulgaris* L.) before and after cooking. The molecular weight of the protein complexes present at neutral pH or after alkaline dissociation of subunits was monitored by size exclusion-high performance liquid chromatography (SE-HPLC) of labeled proteins.

MATERIALS AND METHODS

Dry seeds of a commercial variety of common bean (*Phaseolus vulgaris* L.) were obtained from the local market. Legumes were cooked after being soaked in water at room temperature for 2 h (1:5 w/v for faba bean and 1:4 w/v for the other legume species). Legumes, with the soaking water, were autoclaved for 20 min at 120 °C (1 atm) and then freeze-dried. Raw and cooked legumes were ground in a Cyclotec 1093 Tecator (50 μm). Total protein content (N × 6.25) was determined by the Kjeldahl method (AOAC, 1990) and soluble protein content by the method of Lowry et al. (1951). Protein content, on dry basis, was 26.4 and 26.3% for raw and cooked beans, respectively.

Protein Solubility. A weight of raw and cooked flour corresponding to 8−12 mg/mL protein on a Lowry basis was suspended in water (pH 6.5) or adjusted at pH 12.0 by addition of small amounts of 0.5 N NaOH. The suspensions at pH 6.5 or 12.0 were shaken overnight at room temperature and centrifuged (15 min, 4000*g*). Protein content in the supernatant was assayed by the Lowry method and expressed as a percentage of the total protein content (N × 6.25) of the flour. Samples at pH 12.0 were then gradually brought back to pH 8.5 (12.0 → 8.5), and protein solubility was checked again by the Lowry method (1951) after centrifugation of the extract. Samples were reversed to pH 8.5 to allow proper reaction with the spin label. All samples were diluted to a same protein concentration of 6 mg/mL before spin-label experiments.

Spin-Labeling. The nitroxide radical 3-[(2-isothiocyanoethyl)carbamoyl]-PROXYL (Aldrich Chemicals, Strasbourg, France) was used to label lysine residues. For spin-labeling, 0.5 mL of each sample (raw and cooked bean at pH 6.5 or 12.0 → 8.5) was incubated at 4 °C for 2 h or overnight with 1 mM (final concentration) of the paramagnetic compound in 50 mM phosphate buffer, pH 7.4. The samples were centrifuged at 2000*g* for 3 min. To remove the excess spin label, each sample was loaded onto a PD 10 column (Pharmacia LKB, Montreal, PQ, Canada), previously equilibrated with 50 mM sodium phosphate buffer, pH 6.5 or 8.5, for the samples at pH 6.5 or 12.0 (reversed to pH 8.5), respectively. Eluate (0.4 mL) was collected after passage of 4 mL of buffer (void volume of the column), and electron spin resonance spectra were recorded. After passage through the PD 10 column, the protein concentration of each sample was recalculated by the Lowry method (1951).

Electron Spin Resonance (ESR) Measurements. ESR spectra were collected at the X band (ca. 9 GHz) on a Varian E9 spectrometer interfaced to a Stelar Prometheus Data System for acquisition and handling of the spectra. Measurements were carried out at room temperature in glass capillary tubes. Typically, 9 or 16 scans were collected and averaged for each sample. The concentration of incorporated spin label was estimated by double integration of the ESR spectra vs a standard of free 3-[(2-isothiocyanoethyl)carbamoyl]proxyl at known concentration The mobility of the label was deduced from the H_0/H_1 intensity ratio between the central and the high-field resonances of the triplet. The ratio approaches a value of 1 for a totally free label and progressively increases when the label is immobilized. For a rapidly and isotropically tumbling nitroxide, the H_0/H_1 ratio is directly related to the rotational correlation time τ_R through the empirical formula $\tau_R = 6.5 \times 10^{-10} \Delta B_0 (H_0/H_1 - 1)$, where ΔB_0 is the line width of the central hyperfine (Knowles et al., 1976).

Size Exclusion-High Performance Liquid Chromatography (SE-HPLC). The SE-HPLC of legume proteins was performed on a Waters (Milford, MA) Protein Pak 300SW column of 7.5 × 300 mm. The column was fitted to a Waters M510 HPLC apparatus equipped with a 510 pump model. Proteins were run in 50 mM sodium phosphate, pH 6.5, 0.1 M NaCl, at a flow rate of 0.5 mL/min. A standard curve was obtained by using molecular weight protein markers including blue dextran (2 000 000), thyroglobulin (669 000), apoferritin (443 000), β-amylase (200 000), alcohol dehydrogenase (150 000), albumin (66 000), carbonic anhydrase (29 000), and tryptophan (204). The eluate was monitored at 210 nm with an ultraviolet spectrophotometric detector (PAD Waters model 996), and the chromatograms were analyzed quantitatively through computer deconvolution into the various peaks and measurement of the relative areas. The experiment was performed in triplicate, and data from a representative chromatogram are reported.

Table 1. Solubility of Proteins in Water Extracts of Raw and Cooked Bean at pH 6.5 and 12.0 and at pH 12.0, Reversed to pH 8.5

	total protein[a] (mg/mL)	soluble protein[b] (mg/mL)	solubility (% of total protein)
raw bean, pH 6.5	12.5	8.8	70.0
raw bean, pH 12.0	12.5	11.1	88.5
raw bean, pH 12.0 (to pH 8.5)	12.5	10.6	85.0
cooked bean, pH 6.5	62.5	11.9	19.0
cooked bean, pH 12.0	12.5	10.0	80.0
cooked bean, pH 12.0 (to pH 8.5)	12.5	8.6	69.0

[a] On a Kjeldahl basis. [b] Mean value of three experiments (variability coefficient <5%).

Statistical Analysis. The results were subjected to analysis of variance. The significance of the differences between means was estimated by Student's *t*-test.

RESULTS AND DISCUSSION

Protein Solubility. Protein solubility of raw and cooked common bean (*P. vulgaris*) at pH 6.5 in water was 70 and 20%, respectively (Table 1). As already observed, heat treatment markedly decreased legume protein solubility, likely because of aggregation consequent to protein denaturation (Dench, 1982; Gujska and Khan, 1991; Carbonaro et al., 1997). In either raw or cooked common bean at least 80% protein was solubilized at pH 12.0, as observed with different legume species (Carbonaro et al., 1997). Alkaline conditions were found to be much more effective than acidic conditions in solubilizing protein aggregates from processed legumes (Dench, 1982; Carbonaro et al., 1993, 1997).

Our previous characterization by ultracentrifugal analysis of the protein components formed at pH 12.0 in water following dissociation of heat-induced aggregates of common bean proteins indicated the presence of a single slow species with a sedimentation coefficient of 2.9S (MW 30 000) (Carbonaro et al., 1993). As judged by SDS−PAGE characterization, this component was likely a mixture of several proteins in the MW range 25−43 000. The soluble protein species that were present at pH 12.0 in the water extract appeared to be rather stable because the solubility of raw and cooked bean proteins was maintained when the pH was gradually changed toward the neutral side of the pH range (Table 1). This behavior was expected on the basis of the results of a previous study on *P. vulgaris* proteins (Carbonaro et al., 1993). Indeed, the extent of protein solubility gained at pH 12.0 (about 90 and 75% for raw and cooked bean, respectively) was preserved up to pH

Table 2. Molecular Weight Distribution by SE-HPLC of Protein Components Present at pH 6.5 and 12.0 (Reversed to pH 8.5) in Water Extracts of Raw Bean (Incubation Time with the Spin Label: Overnight)

raw bean pH 6.5			raw bean pH 12.0 (to pH 8.5)		
$t_{R(min)}$	MW	% of area	$t_{R(min)}$	MW	% of area
9.6	1 750 000	2.0	9.5	1 830 000	3.5
12.4	419 000	5.5	11.4	706 600	14.5
14.2	170 300	5.0	13.6	230 300	16.0
16.1	60 000	35.0	14.8	123 900	19.0
17.8	27 000	36.0	17.2	36 800	27.0
20.4	7300	10.0	18.8	16 300	17.0
23.0	2000	5.0	21.1	5100	2.5

Table 3. Molecular Weight Distribution by SE-HPLC of Protein Components Present at pH 6.5 and 12.0 (Reversed to pH 8.5) in Water Extracts of Cooked Bean (Incubation Time with the Spin Label: Overnight)

cooked bean pH 6.5			cooked bean pH 12.0 (to pH 8.5)		
$t_{R(min)}$	MW	% of area	$t_{R(min)}$	MW	% of area
9.5	1 870 000	6.2	9.5	1 900 000	13.5
12.0	505 000	2.3	12.1	500 000	25.0
16.4	54 550	7.0	14.1	180 550	21.5
18.0	24 350	17.0	17.7	28 850	25.0
18.5	18 550	21.0	18.5	18 400	12.0
20.5	6750	22.0	20.7	6150	3.0
21.8	3600	15.5			
22.4	2500	5.0			
24.0	1000	4.0			

2.0, with only a slight inflection around the isoelectric point (pH 4.0). This may reflect some protein precipitation because of electrostatic interactions between oppositely charged groups (lys/arg and asp/glu).

SE-HPLC Analysis. SE-HPLC characterization was performed in order to establish the molecular weight of the protein components present at pH 6.5 and at pH 12.0 (→8.5) in water extracts of raw and cooked bean. The molecular weight and relative percentage of areas of the various species formed at the two pH values for raw and cooked bean at the two different incubation times, 2 h and overnight, with the spin label was very similar. This may suggest that labeling of accessible lysine residues takes place within 2 h and that no other lysines are available for labeling at longer incubation times. Therefore, only the results of the HPLC separation of samples spin-labeled overnight are shown for either raw (Table 2) or cooked bean (Table 3).

At pH 6.5, the main species resolved in the HPLC profile of protein extracted from raw bean showed molecular weights of 60 000 and 27 000 (35% of the total area for either peak) and, therefore, lower than that previously determined when a similar sample was analyzed under the same experimental conditions but without labeling of lysine residues (Carbonaro et al., 1997). In that case, the molecular weight of the main species observed (170 000) was consistent with that of phaseolin, the major storage protein of *P. vulgaris* seed, in the trimeric state (Lawrence et al., 1994). A molecular weight of 60 000, measured in the present study, is within the range indicated for subunits of the 7*S* protein of *P. vulgaris* (Derbyshire et al., 1976). The difference in molecular weight observed might indicate that suppression of positive charge of lysine by spin-labeling results in dissociation of trimers into the monomer subunits. This behavior would not be unexpected because charged residues have been demonstrated to be located on the surface of 11*S* and 7*S* legume protein subunits (Lawrence et al., 1994) and are, therefore,

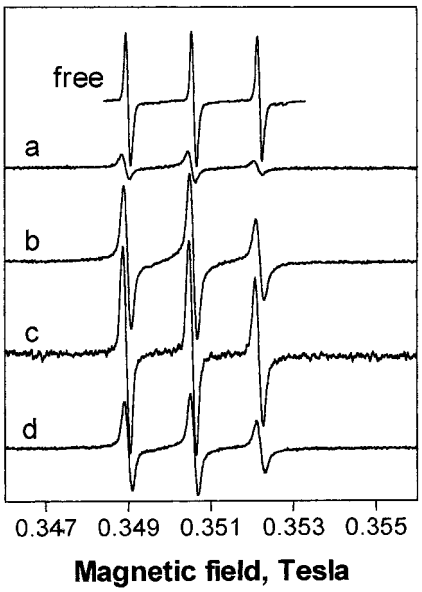

Figure 1. ESR spectra of raw (spectra a, b) and cooked (spectra c, d) beans labeled overnight with 3-[(2-isothiocyanoethyl)carbamoyl]proxyl at pH 6.5 (spectra a, c) or pH 12 (→8.5) (spectra b, d). Spectra have been normalized for the protein concentration. Experimental settings were as follows: central field, 351 mT; scan range, 10 mT; scan time, 10 s; modulation amplitude, 0.01 mT; number of scans, 9; temperature, 298 K. The spectrum of free 3-[(2-isothiocyanoethyl)carbamoyl]proxyl is shown on top for comparison.

involved in the interactions among monomers that are at the basis of their association−dissociation properties (Wright, 1987; Carbonaro et al., 1993, 1997). The role of lysine residues of storage proteins in the mechanism of aggregation that makes possible a proper assembly into protein bodies has already been examined for other food proteins, such as zeins (Wallace et al., 1988). The presence of a low amount of very high molecular weight protein aggregates (about 2 million), previously resolved by SE-HPLC of the water soluble extract of the raw bean at pH 6.5 (Carbonaro et al., 1997), was confirmed in the present study, irrespective of the spin-labeling treatment (Table 2).

As also shown in Table 2, alkalinization of the raw bean extracts induced a significant shift of the chromatogram toward the high MW side, with an almost doubling of the ca. 1.8 million component and a 3-fold enhancement of the second by size peak, which also had a higher MW, from ca. 400 000 to ca. 700 000. This finding strongly suggests that, after dissociation at alkaline pH, subunits of *P. vulgaris* oligomeric proteins undergo a reaggregation process with formation of soluble complexes with a molecular weight much higher than that of the species originally present at neutral pH. Formation of soluble macroaggregates stabilized by electrostatic interactions among constituent subunits has been demonstrated to take place after heat-induced dissociation of 11*S* and 7*S* soybean proteins (Utsumi et al., 1984).

Cooking of legumes and extraction at pH 6.5 showed a decreased solubility of proteins, as already reported in Table 1. Characterization of this extract by SE-HPLC analysis (Table 3) revealed that heat denaturation of the sample at neutral pH mimicked in part the effect of the alkalinization, with a significant increase (from 2.0 to 6.2%) of the highest MW peak. However, the chromatogram indicated that the proteins were in this case distributed over a wider range of molecular weights,

Table 4. ESR Parameters of Proteins in Water Extracts of Raw and Cooked Bean at pH 6.5 and 12.0 (Reversed to pH 8.5) after Incubation with the Spin Label for 2 h or Overnight[a]

	label (μmol/L)	protein (mg/mL)	label/protein (μmol/g)	H_0/H_1
t = 2 h				
raw bean pH 6.5	7.17 ± 0.97	1.80 ± 0.13	3.96 ± 0.60	2.19 ± 0.08
raw bean pH 12.0 (to pH 8.5)	11.32 ± 2.68	2.03 ± 0.23	5.50 ± 0.71	1.93 ± 0.12
cooked bean pH 6.5	6.45 ± 2.67	0.78 ± 0.08	7.50 ± 2.35	1.45 ± 0.24
cooked bean pH 12.0 (to pH 8.5)	6.93 ± 0.58	1.91 ± 0.19	3.71 ± 0.69	1.91 ± 0.06
t = overnight				
raw bean pH 6.5	6.65 ± 0.75	1.91 ± 0.13	3.55 ± 0.65	2.19 ± 0.04
raw bean pH 12.0 (to pH 8.5)	18.79 ± 2.11	2.20 ± 0.27	8.59 ± 0.09	1.96 ± 0.08
raw bean pH 6.5	10.69 ± 2.54	0.96 ± 0.23	14.64 ± 4.97	1.33 ± 0.19
cooked bean pH 12.0 (to pH 8.5)	11.86 ± 1.34	2.22 ± 0.33	5.38 ± 0.18	1.79 ± 0.16

[a] Mean values ± SD of three experiments.

with a definite decrease of the peaks at intermediate (i.e., 20 000−60 000) MW. The combined effects of cooking and alkalinization was dramatic (Tables 2 and 3), with ca. 60% of total proteins eluting with retention times corresponding to MW between 180 000 and 1.9 million.

ESR Spectra of Spin-Labeled Proteins. The paramagnetic compound 3-[(2-isothiocyanoethyl)carbamoyl]-proxyl was covalently linked to lysine residues of proteins extracted from raw and cooked bean at the two different pH values. Since the mobility of the spin label depends on the relative position of protein subunits where lysines are labeled, this should enable acquisition of information about the local environment of lysine residues and, hence, on their accessibility in the various pH-induced association states of *P. vulgaris* proteins.

Free 3-[(2-isothiocyanoethyl)carbamoyl]proxyl in phosphate buffer gave the typical three-line spectra observed for nitroxide radicals (upper spectrum in Figure 1).

Proteins were spin labeled by either 2 h or overnight incubation with 3-[(2-isothiocyanoethyl)carbamoyl]proxyl in phosphate buffer. The results were similar in both cases. Thus, only the ESR spectra obtained after the overnight incubation are reported in Figure 1, where the curves of the spin label bound to raw and cooked bean proteins at pH 6.5 and 12.0 (→8.5) are shown. The ESR data were quantitatively analyzed, and the results are reported in Table 4, where column 1 refers to the integrated ESR signal that, after correction for the protein concentration value (column 2), gives the normalized result (column 3), expressed as "label/protein ratio". Finally, column 4 reports the H_0/H_1 values, which were taken as an index of label mobility (see Materials and Methods). A lower amount of soluble proteins was observed for cooked bean proteins extracted at pH 6.5 (Table 4, column 2). This was not due to treatment with the spin label, since it has been previously reported that cooked bean proteins solubilized at neutral pH are intrinsically less stable than proteins extracted either on raw beans or on cooked beans at alkaline pH (Carbonaro et al., 1993).

It is immediately evident that the mobility of the label was apparently the same for all samples but that of the cooked beans at pH 6.5 (spectrum c), where the line shape was clearly consistent with an essentially free-tumbling nitroxide moiety. As a matter of fact, if H_0/H_1 values were empirically converted into τ_R values (see Materials and Methods), a ca. 3-fold enhancement of mobility resulted for the cooked bean proteins extracted at neutral pH vs all other samples.

When proteins were labeled for 2 h, the label/protein ratio of raw bean sample extracted at either neutral pH or pH 12.0 (→8.5) was not significantly changed ($p < 0.05$) (Table 4). However, when proteins were incubated overnight with the paramagnetic compound, it was increased in raw bean proteins extracted at pH 12.0 (→8.5) compared with those extracted at pH 6.5. Since SE-HPLC characterization indicated no differences in the MW and percentage of the species present at the two incubation times with the spin label, the increase in the label/protein ratio observed at the longer incubation time was guessed to reflect aspecific labeling of protein residues, possibly to hydroxyl groups. Whatever the origin of the higher label/protein ratio for the raw bean at pH 12 than at pH 6.5, the opposite was true for the cooked bean, where the ratio was lower ($p < 0.05$) at pH 12 than at pH 6.5, both in the 2 h and in the overnight incubation with the spin label. The result likely reflects a reduced accessibility of lysine residues in the soluble protein aggregates that are formed after alkaline dissociation of denatured, but not native, subunits of cooked bean. This is also consistent with the impaired mobility of the spin label in cooked bean samples at pH 12.0 (→8.5) indicated by the higher ($p < 0.05$) H_0/H_1 ratio measured in the latter sample than in those at pH 6.5 at either incubation time with the spin label. It is worth commenting, at this point, on the relative significance of mobility and degree of label incorporation. It is clear, from data in Table 4, that neither parameter can, per se, exhaustively describe the environment of a reactive lysine at a molecular level. However, we would simply focus the reader's attention to the fact that, in the case of cooked, but not raw, bean proteins, there is a decrease of labeling extent (i.e., of accessibility) that is concomitant to a decrease mobility when going from neutral to alkaline pH. It is safe to conclude, in this case, that alkalinization of denatured bean proteins has a major impact on the molecular environment of reactive lysines, which have been postulated to be involved in, and even responsible for, the formation of soluble aggregates.

CONCLUSIONS

The results of the present study provide evidence for low accessibility of lysine residues in the aggregates of *P. vulgaris* proteins that are formed after alkaline dissociation of denatured protein subunits.

Although aggregation is also observed to occur in the same conditions for native *P. vulgaris* proteins, lysine environment appeared to be different in the two cases, since only in the aggregates from denatured proteins was accessibility of lysines impaired. This was in agreement with location of lysine at the subunit interface (Lawrence et al., 1994) and higher stability of

complexes that are built up from heat-treated rather than from unheated legume protein subunits, due to the increase in hydrophobic interactions between unfolded polypeptide chains (Hayakawa and Nakai, 1985; Kinsella et al., 1985; Carbonaro et al., 1993).

Trapping of lysine residues inside protein aggregates, by adversely affecting rate and extent of protein digestion by trypsin, may explain limited digestibility of cooked legume proteins in the small intestine (Carbonaro et al., 1998). The composition and properties of aggregates of denatured legume proteins that are formed during in vivo digestion are currently under study.

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