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The effect of added salts on the viscoelastic properties of fish skin gelatin

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Abstract

The effects of various salts on the viscoelastic properties of a class A gelatin (obtained by a mild acid pretreatment) from megrim (*Lepidorhombus boscii*) skins were examined and compared with their effects on commercial tilapia skin gelatin. Although salts generally extended the setting time of gelatins, it was found that the melting temperature was increased considerably by the addition of $MgSO_4$, $(NH_4)_2SO_4$, or NaH_2PO_4. Of all the salts assayed, only $MgSO_4$ improved the rheological characteristics in suitable conditions of pH and ionic strength, which differed between megrim and tilapia gelatin. Notable differences were found in the amino acid compositions of the two species, especially in the hydroxyproline content.

1. Introduction

Gelatin can be used as an ingredient to enhance the elasticity, consistency and stability of food products, and therefore the quality of a food grade gelatin depends to a large extent on its rheological properties. Although mammalian and avian gelatins have been extensively studied, less is known about fish gelatin (Grossman & Bergman, 1992; Gudmundsson & Hafsteinsson, 1997; Holzer, 1996; Kim & Cho, 1996; Norland, 1990; Osborne, Voigt & Hall, 1990). It is generally known that gelatins from warm blooded animals are characterized by having considerably higher melting and gelling points than cold-water fish gelatins (Leuenberger, 1991); moreover, the gels are also stronger, which is directly related to the fact that hydroxyproline content is higher in the former (Ledward, 1992; Norland, 1990). The interest in the utilisation of fish skins lies, not only in the exploitation of by-products, but also, from a socio-cultural standpoint, as an alternative to mammal gelatin. For many applications, good rheological properties are required, and these could be attained by using gelatin-modifying materials. One possible means of manipulating the characteristics of a given gelatin is to trigger interactions by the addition of solutes, for instance, salts (Elysée-Collen & Lencki, 1996). As reviewed by Asghar and Henrickson (1982), electrolytes in general have a decisive influence on the biophysical properties (swelling, solubility, gelation, viscosity and water-binding capacity) of a protein at different ionic strengths and pH values (Hermansson, 1975). There are two points of view as regards possible interaction between collagen (or gelatin) molecules and saline ions. Some workers believe in the possibility of direct-ion binding to the peptide backbone of collagen, while others believe that ions affect collagen folding indirectly by interacting with structurally bound water molecules (Asghar & Henrickson, 1982). According to Fennema (1977), ions may be classified as "water structure formers" or "water structure breakers" on the basis of their ability to alter the net structure of water by its polarizing power, which results in modification of water viscosity. Hydration, caused by the interaction of ions of neutral salts with non-ionic bonds (e.g. hydrogen bonds) of collagen is described as "lyotropic hydration". Thus, lyotropic agents may alter water structure around collagen molecules, interrupt internal hydrogen bonds, or interact with internal hydrophobic bonds by direct binding at some sites of protein chains (Asghar & Henrickson, 1982). It has been stated that the effect of salt concentration on protein stability is very ion-specific, with stabilizing or destabilizing effects typically following the Hofmeister series (Von Hippel & Wong, 1962). However, salts with the same cation but different anions, as well as those with the same anion and different

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cations, did not necessarilly function according to the lyotropic order (Hamm, 1958).

The effect of different salts on the rigidity or melting temperature of animal gelatins has been known for a long time (Harrington & Von Hippel, 1961). Other substances, such as dextran dialdehydes, have been tested to improve crosslinking of gelatin (Schacht, Nobels, Vansteenkiste, Demeester, Franssen & Lemahiev, 1993). More recently ammonium sulphate has been found to reduce the solubility of gelatin as a consequence of both, protein and salt competing for water to hydrate (Elysée-Collen & Lencki, 1996). However, little information is available in relation to the effect of salts in food grade gelatins obtained from fish skins, which differ greatly from gelatins of animals.

The aim of this work was to examine the effect of several salts, at high (0.5 M) and low (0.1 M) concentration, and at two pH levels (pH 5 and 8), on the viscoelastic properties of a class A gelatin (obtained with a mild acid pretreatment) from megrim (*Lepidorhombus boscii*) skins. This species may be classified as half-way between a cold water species and a typical warm water species such as tilapia, which has been used for several fish gelatin patents (Grossman & Bergman, 1992; Holzer, 1996). A comparison was made between megrim and tilapia gelatins on the basis of their amino acid composition and viscoelastic properties, and the effect of addition of the different salts was also compared.

2. Materials and methods

Fresh megrim [*Lepidorhombus boscii* (Risso)] skins were obtained from a local market in Madrid, and were stored at −20°C until use. A commercial tilapia (*Oreochromis* spp) skin gelatin was supplied by Croda Ltd. (UK). All reagents used were analytical grade.

2.1. Cleaning of megrim skins

Thawed skins were washed with tap water (1:6 w/v) in a Stephan homogenizer (position II, very strong stirring) (Stephan Model U M5, Stephan u. Söhne GmbH & Co., Germany) at 5°C for 10 min, and were rinsed with abundant running tap water. Skins were further cleaned with 0.8 M NaCl (1:6 w/v), again in the Stephan at 5°C for 10 min, and were rinsed with abundant running tap water. This step was repeated three times. Excess water was removed by draining the cleaned skins and squeezing in a manual press.

2.2. Gelatin extraction procedure

Cleaned dark megrim skins were stirred with 0.2 M sodium hydroxide (1: 6 w/v) at 5°C for 30 min (repeated three times). Samples were drained and rinsed with tap water after each step. Skins were swollen with 0.05 M acetic acid (1:10 w/v) at room temperature for 3 h, rinsed with tap water and then extracted with distilled water overnight at 45°C. The dark pigmented thin layer was removed and the remaining mixture was filtered in a Büchner funnel with a Whatman no. 4 filter paper and air-dried in a convection oven at 40–42°C until the final moisture content was less than 15%.

2.3. Viscoelastic properties measurement

Dynamic viscoelastic studies were performed on a Bohlin CSR-10 rheometer rotary viscometer (Bohlin Instruments Ltd., Gloucestershire, UK) using a cone-plate geometry (cone angle 4°, gap = 150 mm). Temperature ramps were implemented from 50 to 5°C, and back to 50°C, at a scan rate of 0.5°C/min, frequency 1 Hz and oscillating applied stress 3.0 Pa. Dry powder was dissolved in water or in different salt solutions, at 45°C (at 6.67% concentration) before the start of the test. The salt solutions assayed were: NaCl, $MgCl_2$, $MgSO_4$, $(NH_4)_2SO_4$ and NaH_2PO_4, at 0.5 M and 0.1 M (pH 5 and pH 8, in each case). Samples were subjected to the oscillatory test immediately after dissolution. The melting temperature was taken as the point at which the phase angle peaks immediately after a sharp increase. Setting time (gel onset time) was determined as the time in minutes elapsing between last temperature of maximum phase angle and first temperature of minimum phase angle (gelling point)). In order to allow suitable gelling in all studied samples, reference G' and G'' (Pa) values were taken at 5°C to compare characteristics at a given standard temperature. The results obtained were averages of four determinations. The error in reproducibility of the parameters considered in different determinations of a single sample was 6% or less.

2.4. Amino acid composition

For the analysis of amino acids, the dry powders were reconstituted with distilled water at 1 mg/ml. Approximately 50 μg of sample was treated by acid hydrolysis (HCl 5.6 M) at 108°C for 18 h, and injected into a Beckman 6300 analyzer (Beckman Instruments Inc., Palo Alto, CA, USA). Analyses were carried out in duplicate and results were expressed as number of residues per 1000 residues.

3. Results and discussion

A comparison was made of viscoelastic properties of megrim gelatin powder dissolved in different saline solutions, at both pH 5 and 8, and at 0.5 and 0.1 M salt concentration. Fig. 1 shows the elastic modulus (G'),

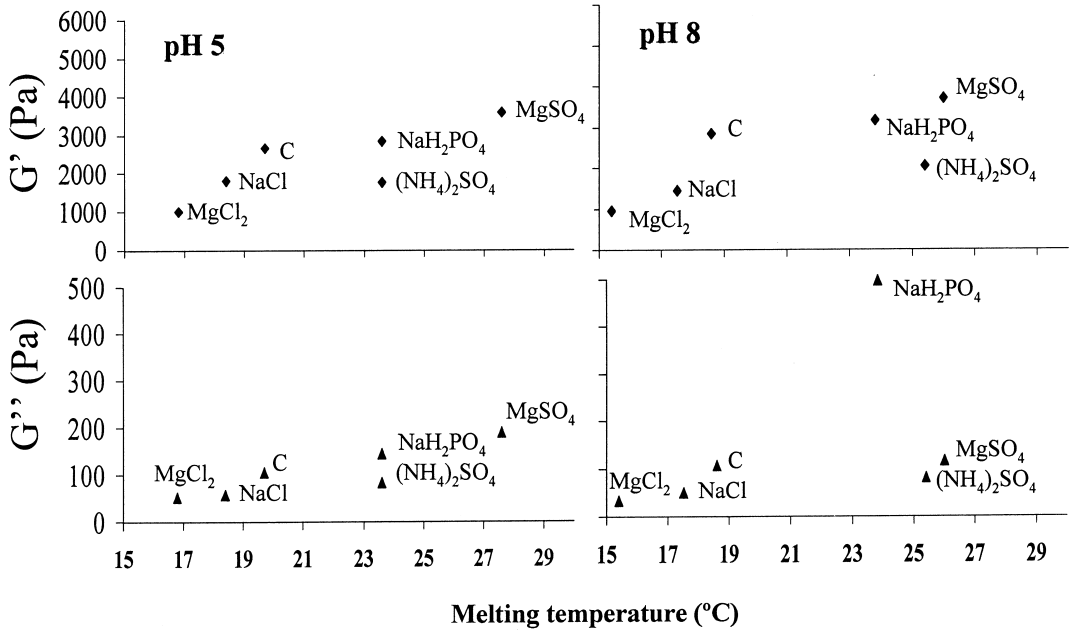

Fig. 1. Elastic modulus (G') and viscous modulus (G''), measured at 5°C, plotted as function of melting temperature, of megrim skin gelatin dissolved in several salt solutions at 0.5 M concentration, at both pH 5 and 8.

viscous modulus (G'') (both measured at 5°C), and melting temperature, of gelatin dissolved at 0.5 M salt concentration (pH 5 and 8). A class A gelatin (mild acid pretreatment) will carry a net positive charge in all food uses, irrespective of the pH of the medium (Stainsby, 1987), therefore the gelatin without added salt (C) was practically not affected by pH. There was a notable increase in the melting temperature with respect to the gelatin without added salt when sulphates and sodium phosphate were added. On the other hand, the chloride salts (NaCl and $MgCl_2$), especially the latter, produced a lower melting temperature. Saline ions can interact with the charged polar groups of the protein, or else they may remain free and mobile in the aqueous phase, depending on the pH of the medium, the nature of the ions or the protein (Fennema, 1976). Because of their larger size, sulphates and phosphates remain further from the centres of positively-charged protein chains, whereas ions of smaller radius (chlorides) can approach more readily and hence can interact with them more easily. The former are more likely to interact with the surrounding water and produce greater screening of electrostatic interactions. As a result they open up the protein chains more and thus increase the likelihood of a suitable formation of useful strong junctions which, as reviewed by Ledward (1992), dictate the melting point. Multiplication of junction points through salt bridges has been reported (Tar & Wolfram, 1979). Only gelatin dissolved with $(NH_4)_2SO_4$ reached a higher melting temperature at pH 8 than at pH 5. By decreasing the H^+ content of the medium, the ammonium ion, because of both its positive charge and its large size, can favour electrostatic repulsion between peptide chains, leading to a higher degree of protein unfolding.

Although the addition of $MgSO_4$ slightly increased the elastic modulus G', in general no evident improving effect in gel development by salts was observed. Salts have been reported to destabilize gelatin structure (Slade & Levine, 1987), probably as a direct consequence of both protein and salt competing for water to hydrate (Elysée-Collen & Lencki, 1996). As noted by Stainsby (1987), promotion of helix formation by screening of ionic interactions by salts, does not necessarily result in stronger gels and, therefore, there must be another factor involved. A weak gel may have been formed when the initial nucleation sites have not been able to anneal themselves into their most stable conformation to encourage further growth of these zones on subsequent cooling (Ledward, 1992), and this is directly related to the effect of the injected saline ions. Possible loss of gel rigidity by most salts has also been explained in terms of an appreciable decrease in specific levorotation (Harrington & Von Hippel, 1961). The NaH_2PO_4 sample, in particular presented an exceptionally high viscous component G'' at pH 8, which did not correspond to a high G'. At such high pH, interaction between the multivalent anion and the positively-charged protein chains could have been promoted, preventing these from contributing to gel network formation, which would increase the viscous component.

When the salt concentration was reduced to 0.1 M (Fig. 2), the $MgSO_4$ sample, at pH 5, was the only one that increased the melting point with respect to the control, although the value (25°C) was slightly lower

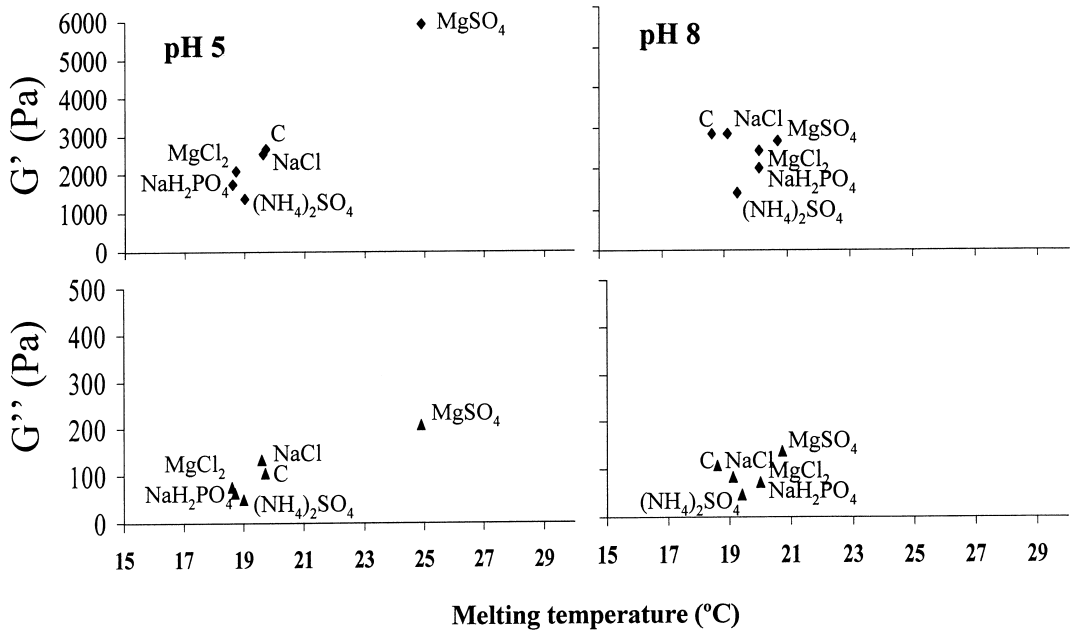

Fig. 2. Elastic modulus (G') and viscous modulus (G''), measured at 5°C, plotted as function of melting temperature, of megrim skin gelatin dissolved in several salt solutions at 0.1 M concentration, at both pH 5 and 8.

than with 0.5 M concentration (28°C). In this case, the $MgSO_4$ improved gel network formation through a pronounced increase in G' and to a lesser extent in G''. The lower ionic strength (0.1 M instead of 0.5 M) would appear to be more suitable in the case of $MgSO_4$ for promotion of useful junctions by correct protein unfolding without distorting the subsequent assembly of the chains into collagen-like helical rods. $(NH_4)_2SO_4$, on the other hand, led to the lowest G' and G'' at both pH 5 and 8. $(NH_4)_2SO_4$ and NaH_2PO_4 were the only ones to produce lower G' and G'' at 0.1 M than at 0.5 M concentration.

The addition of salt considerably prolonged the setting time in most cases when compared to the control (Table 1), due to the destabilizing effect on gelatin structure (Slade & Levine, 1987). In general, setting time tended to be shorter at pH 8 than at pH 5, except for gelatin with added $(NH_4)_2SO_4$. This could be explained by the fact that, at pH 8, the gelatin is close to its isoelectric point, where protein-protein interactions predominate, so that the gel forms more rapidly. As reported above, at high concentration (0.5 M) and pH 8, $(NH_4)_2SO_4$ led to greater protein unfolding, which considerably retarded subsequent gelation. $MgSO_4$ at both salt concentrations, and $MgCl_2$ at 0.5 M and pH 5, provided the highest setting time. The delay in gelation by magnesium salts, especially at 0.5 M concentration, could be explained on the basis of the Hofmeister lyotropic series, as a salting-out effect, and also by the fact that it can interact with various negatively-charged groups in the gelatin polypeptide chains, retarding gelation.

As observed for the melting temperature, magnesium salts and NaH_2PO_4 showed the highest gelling temperatures, especially at 0.5 M (pH 5 and 8) (Table 1). $MgSO_4$ in particular produced a gelling temperature of 18°C when added to gelatin at 0.5 M concentration and pH8; however, when salt concentration decreased to 0.1 M, the gelling temperature was considerably reduced to 14°C.

Of all the salts assayed, $MgSO_4$ at the right ionic strength and pH conditions was the one that most favoured viscoelastic properties of gelatin. Some properties, such as melting point, have been clearly attributed to the sulphate ion, while others, like setting times, have been associated in some way with the Mg^{2+} ion. However, the fact that both ions were located on the right extreme or at the end of the Hofmeister or lyo-

Table 1
Setting time and gelling temperature of megrim skin gelatin dissolved in several salt solutions at 0.5 and 0.1 M concentration, and at pH 5 and 8

	Setting time (min)				Gelling temperature (°C)			
	pH 5		pH 8		pH 5		pH 8	
	0.5 M	0.1 M	0.5 M	0.1 M	0.5 M	0.1 M	0.5 M	0.1 M
Control	12		12		13		13	
NaCl	16	17	13	13	12	12	12	13.5
$MgCl_2$	18.5	15	16	16	8.5	12	8	13
$MgSO_4$	22	19	18	12.5	16	15.5	18	14
$(NH_4)_2SO_4$	14	17	21	15	16.5	13.5	14.5	13.5
NaH_2PO_4	17	13	13	15	16.5	13.5	17.5	14

Table 2
Viscoelastic properties of tilapia skin gelatin dissolved in several salt solutions at 0.5 and 0.1 M concentration, and at pH 5 and 8

		Elastic modulus G' (Pa)[a]		Viscous modulus G'' (Pa)[a]		Setting time (min)		Melting temperature (°C)		Gelling temperature (°C)	
		pH 5	pH 8	pH 5	pH 8	pH 5	pH 8	pH 5	pH 8	pH 5	pH 8
Control		4543	3339	676	66	9	10	19	19	15	16
NaCl	0.5 M	2357	2084	145	48	7	9.5	17	16	13	12.5
	0.1 M	3128	3003	132	103	10	7	18	18	14.5	14.5
$MgCl_2$	0.5 M	1452	1426	173	60	15	10	17	14	9.5	9.5
	0.1 M	3308	3079	115	74	8	8.5	18	18	14.5	14
$MgSO_4$	0.5 M	5126	4930	205	353	14	9	24	23	19	19
	0.1 M	3874	3409	84	52	9	8	20	20	15.5	15.5
NaH_2PO_4	0.5 M	4286	nm[b]	195	nm	11.5	nm	22	nm	18.5	nm

[a] Measured at 5°C.
[b] nm, not measured.

tropic series is important, given that according to Steinberg, Harrington, Berger, Sela and Karchalaski (1960), it is precisely the ions located on the left extreme that most favour a disordered triple helical structure with diminished rheological properties.

The viscoelastic properties of megrim gelatin were compared with those of a commercial fish skin gelatin from tilapia (see Table 2). At pH 5 and 8, the tilapia gelatin, without added salt, presented a similar melting temperature to that of megrim gelatin, but G' was higher, especially at pH 5. Tilapia gelatin seemed to be more affected than megrim by pH, and its G'' value was considerably higher at pH 5 than at pH 8, probably because the latter may be near the isoelectric point. At both pHs, tilapia gelatin had a slightly shorter setting time and higher gelling point than megrim. As regards salt concentration, the assayed salts had little effect observable when used at 0.1 M, in contrast to 0.5 M. As in the case of megrim, $MgSO_4$ greatly increased the melting point at 0.5 M salt concentration (pH 5 and 8), but not at 0.1 M. Unlike megrim gelatin, there was an appreciable increase in G' only when $MgSO_4$ was added at 0.5 M and pH 8. This difference in the behaviour of $MgSO_4$ in megrim and tilapia according to pH and concentration, was attributed directly to differences in the conformation of the protein molecules of the two fish species, which in turn depend on other physicochemical factors such as the isoelectric point and the amino acid composition. $MgSO_4$ considerably increased the G'' at pH 8 and 0.5 M salt concentration, where the G'' of the control sample was particularly low. Chloride salts, as observed for megrim gelatin, seemed to hinder gelation since they considerably reduced G' and G'' values with respect to the control sample, as well as the melting and gelling points. The setting time at pH 5 was noticeably increased by magnesium salts at 0.5 M concentration but, in this case, unlike megrim gelatin, no effect was found at pH 8.

The differences found between megrim and tilapia gelatin could be in part attributed to specific variations in amino acid composition (Table 3). Tilapia gelatin had a considerably higher pyrolidine content (hydroxyproline + proline) than megrim. According to Ledward (1986), this would explain the higher G' and G'' attained by tilapia as compared to megrim gelatin, given that the amount of triple helical structure, which is essential to improve rheological properties of gelatin, are greatly dependent on the amount of pyrolidine residues. Where the two species differ substantially, however, is in hydroxyproline content, which is much higher in tilapia gelatin. According to Ledward (1992), although proline is important, hydroxyproline is believed to be the major determinant of stability due to its hydrogen bonding ability through its –OH group. A bivalent cation, such as Mg^{2+}, can form coordinate

Table 3
Amino acid composition of megrim and tilapia skin gelatins

Aminoacids	No. residues/1000 residues	
	Megrim	Tilapia
Hyp	60	79
Asx	49	48
Thr	22	24
Ser	47	*35*
Gix	70	69
Pro	117	119
Gly	*353*	347
Ala	119	123
Val	16	15
Met	14	9
lie	7	8
Leu	21	23
Tyr	3	2
Phe	14	13
His	8	6
Hyl	6	8
Lys	26	25
Arg	47	47

links involving this –OH group (Gustavson, 1956). This would explain, in part, the special ability of $MgSO_4$ to increase the melting temperature and G' when added at high concentration and pH 8, where the protein was expected to be less unfolded because of its likely proximity to the isoelectric point. This effect, however, was not evident with $MgCl_2$, certainly due to the predominantly hindering effect of the large monovalent anion (Cl^-). Although the amount of hydrophobic residues is similar in megrim and tilapia (around 30%), the latter contained more alanine which, together with proline and hydroxyproline, is found in non-polar regions where tripeptides of the type glycine-proline-R (where R is any amino acid including hydroxyproline) predominate (Ledward, 1986).

Megrim gelatin, however, presented a larger number of serine and methionine residues, of which the latter is interesting for its sulphur atom. Through its terminal OH group, serine can favour interaction with divalent cations such as Mg^{2+} (Gustavson, 1956) which, on the basis of the lyotropic series, would encourage a more ordered triple helical structure (Steinberg et al., 1960).

4. Conclusions

The addition of $MgSO_4$, $(NH_4)_2SO_4$ or NaH_2PO_4 at high concentration (0.5 M) to megrim gelatin was critical in considerably raising the melting point, whereas chloride salt acted to reduce it. At 0.1 M concentration and pH 5, $MgSO_4$ was the only salt that improved melting point and elastic modulus although with the drawback of considerably prolonging the setting time.

A commercial gelatin from tilapia presented a similar melting temperature to megrim gelatin, but a higher G', which was related to the higher hydroxyproline content in tilapia, and to some extent also to its higher alanine content. $MgSO_4$ also increased the melting temperature and G' in tilapia gelatin but, unlike megrim, it did so at high concentration (0.5 M) and pH 8, which revealed conformational differences among the molecules in both species.

It is therefore possible to improve the functional properties of fish gelatins such as megrim skin gelatin to achieve characteristics similar to those of gelatins from warm-blooded animals, chiefly with regard to melting point, by the addition of neutral salts in appropriate conditions of pH and ionic strength.

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