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Lysine Availability in Protein–Alginate Ester Gels

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ABSTRACT

The reduction in available lysine through the interaction of ε-amino groups with propylene glycol alginate to form thermostable gels was investigated using three methods—FDNB, formol titration and dye-binding. Formol titration is preferred for reliability and rapidity. The available lysine content was reduced by at most 18% for disordered proteins (gelatin, caseinate) and from 11 to 18% for structural proteins (whey, soya, egg albumen). The gels are readily attacked by the main digestive proteolytic enzymes, pepsin and trypsin.

INTRODUCTION

In many foods, proteins and polysaccharides interact non-covalently, through hydrogen bonding, hydrophobic bonding and, if the polysaccharide carries ionising groups, by ionic bonding. Such interactions have no effect on the lysine availability of the protein. However, this availability is impaired when the lysyl residues are involved in chemical reactions (covalent bonding) with active groups on the carbohydrate, as in non-enzymic browning reactions. In nutritional terms this reduces the food value of the protein.

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The deliberate linking of polysaccharide to protein, via covalent bonding, to form edible gels has become of interest in recent years because of the need to form attractive and palatable foods from unattractive protein waste. One example is the patent (Unilever, 1976) for making fish fingers or fish cakes from unrefined, comminuted 'meats' using alginate ester. Such gels are particularly stable during cooking and on storage at sub-zero temperatures (Mohamed & Stainsby, 1984). The main crosslinking reaction is the amidation of an ester group on the polysaccharide with an ε-amino group and the protein (Wilson, 1978), thus reducing the availability of the nutritionally important amino acid lysine. Only a very limited number of crosslinks needs to be formed to create the three-dimensional network which gives the thermostable gel (Stainsby, 1980) but it is probable that, as with any tanning reaction, only a small proportion of the bonds that are formed are crossbonds. Physical methods, which determine only the extent of crosslinking, are thus likely to give serious underestimates of the total extent of reaction and thus the total loss of available lysine. Chemical methods must therefore be used, and in this paper three are examined—the fluorodinitrobenzene (FDNB) method, formol titration and a dye-binding method. The first two methods involve the well-known reactions of amino groups with 2,4-dinitrofluorobenzene and formaldehyde, respectively. The third method relies on the affinity between an anionic dye and charged amino groups. The FDNB method is widely used as a standard to assess other methods for determining available lysine. The other two methods are more rapid and convenient. None, however, are entirely straightforward in this application as the gel is stable and restricts the access of chemical probes for the residual available groups within it. However, only minor modifications to established methods have been found necessary to provide reliable measures of the lysine availability, and these modifications are described.

MATERIALS AND METHODS

The alginate ester was Manucol E/RE, supplied by Alginate Industries Ltd.

All the proteins were in the form of fine powders, as described by Mohamed and Stainsby (1983). Other chemicals were obtained from BDH, if possible as Analar grade.

Preparation of the gel

Equal volumes of 6% (air dry) protein and 4% alginate ester were vigorously mixed at room temperature and the resultant gel allowed to mature for 2–3 h, to reach full strength (Mohamed & Stainsby, 1983), before the loss of ε-amino groups was evaluated. The protein was dissolved in bicarbonate/carbonate buffer, pH 9·6, and the ester first dissolved in water and then neutralised with strong alkali immediately before mixing. In this way the premature loss of reactive ester groups and the formation of unwanted CO_2 bubbles were minimised.

FDNB assay

The essential principle of the method, introduced in 1945 by Sanger, is the addition of the reagent to free amino groups to give coloured adducts (DNP derivatives) which are sufficiently stable during subsequent acid hydrolysis of the protein. After hydrolysis they are separated chromatographically and determined spectrophotometrically at 435 nm.

Reaction of the DNP-amino acids, after hydrolysis of the protein, with methoxy carbonyl chloride was introduced by Carpenter (1960), so that only DNP-lysine and DNP-hydroxylysine are ether-soluble. This facilitates their determination for nutritional assays, and the clean separation of these derivatives was further improved by Booth (1971), whose procedure has been used in the present assays. A small proportion of the DNP-ε-amino acids is inevitably still lost during the prolonged acid hydrolysis and the correction factors for this, determined by Booth, have been used. That is, the recovery of animal protein is taken as 93% and for soya isolate a recovery of 90% is used.

The procedure was first carefully checked using a gelatin gel without any polysaccharide present, with due allowance for the moisture and ash of the air-dry powdered protein. Then, since the presence of starch is known to reduce the recovery of lysine and so give too low a value (El Nockrashy, 1965), the effect of alginate had to be evaluated. This was achieved by completely de-esterifying the ester in the pH 9·6 buffer before mixing it with the protein solution—no gel could then form—and then determining the ε-amino acid content of the mixture. It was assumed that the apparent loss of lysine which resulted from the presence of the alginic acid in the mixture, would also occur in a gel as it contained the same proportion of polysaccharide to protein.

Formol titration

In its simplest form, the formol titration involves adding standardised alkali to a protein solution until phenolphthalein changes colour, adding neutralised formaldehyde and then titrating the acid which is produced to the same end-point as before. The additional alkali, after adding formaldehyde, is taken to be equivalent to the ε-amino groups present initially; i.e. none of these groups has been titrated before adding formaldehyde.

Corbet *et al.* (1964) have shown that the colour change of the indicator can easily be masked, so that spurious results are obtained. A titration was therefore used, and the gel homogenised to facilitate the release of acid by formaldehyde. Kenchington (1960) drew attention to the necessity for first neutralising the formaldehyde which frequently contains acidic impurities, and to ensure that no error arose from this source the sample was brought to pH 9·0 just before use.

The choice of pH for the 'end-point' is not usually easy, though there is no problem with formaldehyde-treated gelatin as the lack of phenolic residues then ensures that there is a dramatic increase in pH for a very small addition of alkali, once the ε-amino groups are titrated. This begins at pH 9, and this value was chosen for all the proteins studied. The situation is more complex in the initial, wholly aqueous titration, since the titrating regions for histidine and α-amino groups overlap with that for ε-amino groups. The arbitrary value of pH 9 was again used, though this may mean that some of the ε-amino groups are not counted in the subsequent formol titration.

Two further problems remain, both of which are peculiar to the gels being studied—the buffer and the unreacted ester interfere with the titration and are not easily removed from the gel. The buffer was converted to sodium chloride by adding 0·1 M HCl to the homogenised gel (30 ml for 10 ml buffer). Though this produces a CO_2-foam it is readily broken by immersing the system in a 90 °C bath for just a few minutes. All the unreacted ester was then converted to uronic acid by adding alkali (10 ml 0·1 M NaOH for 0·4 g alginate ester) and allowing de-esterification to proceed for 30 min at room temperature. Then the homogenised dispersion was carefully brought back to pH 9 and the formol titration begun. In this way the uronate ions play no part in the formol titre. It is assumed that no lysine is made available when the buffer and ester are being 'neutralised'.

The formalin solution was 40% formaldehyde, and standardised 0·1N NaOH was used for subsequent formol titration.

Dye-binding

The basic amino acid residues (histidine, arginine, lysine/hydroxylysine, i.e. HARL) in proteins bind anionic dyes and the consequent decrease in dye colour can be used to monitor the overall HARL content. Unfortunately the binding is not purely electrostatic, and the number of dye molecules bound to each residue varies with the nature of the dye and the excess concentration of dye in equilibrium with the complexes. Hurrel and Carpenter (1975) have shown that the association is close to one molecule of dye per basic residue, for several proteins, when Acid Orange 12 is used in a particular oxalic acid–acetic acid–phosphoric acid buffer, pH 1·25. If the content of lysyl/hydroxylysyl residues is required, then these authors showed that a difference technique could be used, i.e. binding by HARL compared with that by HAR, determined after L had been blocked by propionylation.

Thus, equal weights of protein were placed into reaction vessels X and Y. The lysine in vessel X was propionylated, following Hurrel and Carpenter (1975), for 20 min at room temperature. Then stock dye/acidic buffer solution was added to both vessels and, after shaking vigorously for a further 20 min, a portion of each dye solution was removed by filtration through glass fibre and its absorbance determined at 480 nm. The amount of dye bound (by HARL in one vessel and HAR in the other) was then deduced using a standard curve of absorption against dye concentration. The stock dye solution (concentration 3·70 mM) is so intensely coloured that the standard curve (1·00–3·70 mM against 3·70 mM as reference) is highly curved. This does not facilitate good precision, but it is more than adequate to demonstrate that for casein and gelatin the recommended time for reaction with the dye is inadequate and the time for propionylation is also too short, particularly for soya isolate. Walker (1979*a*, *b*) has shown similar shortcomings for dye binding to leaf proteins.

For the present purpose the propionylation step is unnecessary as only differences in lysine content are required. Furthermore, if the filtrate, after reaction, is diluted 100 times with distilled water the absorbance is linearly related to dye content over the range 0–0·05 mM. These advantages were utilised, and the time for reaction with the dye extended to 40 min.

RESULTS AND DISCUSSION

FDNB method

The results are summarised in Table 1, from which it is seen that the values for gelatin and casein before reaction with alginate ester (column G) are in good agreement with literature values (column L). There is no literature value for Promine D, but the value given in brackets in the table has been calculated from the published compositions of the main component proteins and their proportions in this sample as determined by ultracentrifugation. This calculated value agrees well with the experimental content via FDNB.

TABLE 1
Values of Available Lysine and Hydroxylysine Residues in 10^5 g of Various Protein and Protein–Alginate Gels

	L	*G*	*F*	*G–F*	*G′*	*Corrected G′*
Gelatin	38·5	38·5	30·4	8·1	24·6	32·7
Casein	53·2	54·5	45·9	8·6	36·2	44·8
Soya proteins	41·2	42·0	33·7	8·3	25·8	34·1

When alginic acid was added, lower apparent lysine values were found, column F. The difference between the true and apparent contents (8·6 mol per 10^5 g casein) is added to the observed lysine content of the gel (column G′) to yield the corrected content in the gel (column corrected G′). This is the figure used in Table 2, where the FDNB values are compared with those found by formol titration.

Formol titration method

Despite the problems associated with the carbonate/bicarbonate buffer and with the alginic acid/ester, the results obtained by formol titration are in reasonably good agreement with those for the FDNB method. As the formol is a much simpler procedure it has been used to study other proteins and their gels with alginate ester (Table 2). The reproducibility of the method was better than 0·05 ml 0·1N NaOH, i.e. 1 mol lysine per 10^5 g protein.

TABLE 2

Reduction of Available Lysine After Formation of Alginate Gels

(*a* Calculated from data given by the manufacturer. *b* Obtained from standard textbooks)

	Moles lysine per 10^5 g protein	*Millilitres of 0·1N NaOH Formol*		*Per cent lysine involved in interaction*	*FDNB method*		*Moles Hist. Arg. Lys. (HARL) per 10^5 g protein*		*Dye binding capacity. Residues HARL/Dye*
		Theory	*Titration*		*Mole lysine per 10^5 g protein (FDNB)*	*Per cent lysine*	*Theory**	*Dye-binding*	
Air-dried gelatin	38·5	1·90	1·80		38·5		94·6	46·3	0·49
Gelatin–PGA	*b*		1·50	17·0	32·7	15·0	88·8	42·1	0·47
Casein (0)	53·2	2·65	2·80		53·2		101	67·9	0·67
Casein–PGA	*b*		2·30	18·0	44·8	18·0	89·7	57·0	0·64
Soya isolate (Promine D)	41·2	2·65	2·80		42·0		129	126	0·98
Soya–PGA			1·80	18·0	34·1	19·0	121	59·1	0·49
WPC Solac	51·7	2·60	2·60						
Solac–PGA	*a*		2·25	13·5					
WPC 64	56·8	2·85	2·50						
64 PGA	*a*		2·20	12·0					
WPC 56	54·9	2·75	2·50						
56 PGA	*a*		2·20	12·0					
WPC 4			3·50						
Whey–PGA			3·05	13·0					
Egg albumen	40·3	4·45	4·55						
PGA–albumen			4·05	11·0					
PGA–albumen + 0·5 ml 1·0M NaOH	*b*		3·75	18·0					

* Calculated from FDNB results.

The absolute lysine content, by formol titration, depends on the choice for the starting point and end-point of the titration. Here pH 9·0, determined with a meter, was used throughout and for gelatin the formol method gave a rather higher value than the FDNB method. Very much larger differences would have been found if phenolphthalein had been used instead of a meter, since the pH for the colour change of this indicator was found to be 9·25 in the aqueous solution of the protein and 8·6 in the formol solution. The use of the indicator would thus have shown a lower content of free lysine than the values in Table 2. It is also of interest that a significant volume of alkali was required to take the formol from pH 7 to pH 9: 0·25 ml, in fact. This would have counted as additional free lysine and emphasises the need for strict control of the pH of the formol.

Dye-binding method

The results in Table 2 clearly show that although the extent of dye-binding is as expected for soya isolate it is very considerably lower for gelatin and caseinate. It is unlikely that a longer time of contact between the protein and the dye would raise the binding capacity to the desired value of unity, and it is not very practicable to make a substantial increase in the free dye concentration (to force more on to the protein). If it is assumed that the binding to the free positively charged sites in the gel mirrors the binding to these sites on the protein alone (i.e. before reaction with alginate later) then the HARL values of 42·1 and 57 in Table 2 suggest that 22% and 30% of the lysine is no longer available in the gelatin and caseinate gels respectively. These values are very substantially greater than those found by the FDNB and formol methods. The reasons for the difference have not been identified. The discrepancy for soya–alginate ester gel is even more marked.

Although the procedure used is simpler than that of Hurrel and Carpenter (1975), by omitting the propionylation stage, and is more soundly based, using dilution to avoid a sharply curved relationship of dye concentration to absorbance, it nevertheless contains an inherent weakness—the small change in dye binding has to be assigned to only a proportion of the sites, assuming that an average dye-binding capacity prevails. This cannot lead to precise estimates for the loss of lysine. Furthermore, some of the proteins that were examined—but not any of the results in Table 2—produced a haze with the dye. This could not be removed by filtration through porosity 4 glass sinters, nor by

centrifugation at 2×10^3 g. It is thought to be due to hydrophobic interaction between dye and protein-bound dye.

It has to be concluded that this apparently promising method has not proved satisfactory for protein/alginate ester gels.

CONCLUSIONS

Of the three methods examined to determine the available lysine contents of protein–alginate ester gels the most satisfactory is the formol titration. This is a rapid and reliable method, provided care is taken to remove interfering buffers (here bicarbonate/carbonate and alginate ester). By comparison the FDNB method is very laborious, and requires a significant correction to allow for the effects of the very large content of polysaccharide in the sample. The dye-binding procedure of Hurrel and Carpenter (1975) was unsatisfactory.

Only a fairly small fraction of the available lysine becomes involved in covalent bonds with alginate esters when a gel forms. The *total* loss for the disordered proteins, gelatin and casein is less than one-fifth of the ε-amino groups, whilst for the highly ordered proteins (soya, whey, egg albumin) about one-seventh of these groups become unavailable. Most probably only a small fraction of the unavailable groups is involved in creating the gel network, i.e. in bridging protein to protein via polysaccharide, or vice versa.

The large fraction of available lysine suggests that this type of gel should still be attacked readily by digestive enzymes (and the amide bond may well be cleared by some of the enzymes which are present naturally, though this is not yet established). Of the two main digestive proteases, pepsin and trypsin, it is trypsin which cleaves peptide bonds specifically adjacent to free lysine (and arginine) residues. Gelatin alone is readily broken down by trypsin at pH 8 and 38 °C, and the gelatin in an homogenised gelatin–alginate ester gel is also hydrolysed rapidly. Other protein–alginate ester gels behave similarly, and all are attacked readily by pepsin at pH 2. Quantitative evaluation of the extent of breakdown is not easy (Mohamed, 1981). Alginate, on the other hand, is not digested by humans, though some depolymerisation no doubt takes place in the mildly alkaline conditions that are found after leaving the stomach.

The limited loss of available lysine and the ease of hydrolysis by digestive proteases, support the view that protein–alginate ester gels

could form a useful part of the human diet. If made from waste protein, as an essential part of attractive fabricated food, they would help to combat the current world shortage of edible protein.

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