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Synthesis of some sulphydryl dipeptides with novel anti-browning properties

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The synthesis of cysteine-containing dipeptides (with glycine, leucine, β-alanine, α-aminobutyric acid, glutamic acid, valine and methionine) is described. The kinetics of their reaction with 3-deoxyhexosulose as well as their anti-browning potential are assessed. Such dipeptides could form the basis of effective replacements for sulphite as anti-browning agents but, for food uses, their safety would need to be established.

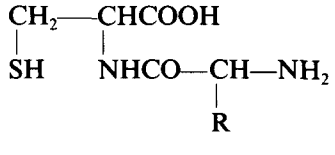
INTRODUCTION

The key intermediate in colour formation in the Maillard reaction between glucose and glycine is 3-deoxyhexosulose, DH. This dehydrates to 3,4-dideoxyhexosulos-3-ene, DDH, before extensive condensation with amine-containing compounds to form coloured high molecular weight products, i.e. melanoidins (McWeeny *et al.*, 1974).

The ability of sulphite species, S(IV), to inhibit the Maillard reaction is the result of a nucleophilic reaction of the additive with DDH (McWeeny *et al.*, 1974; Wedzicha, 1984), but any powerful nucleophile is potentially capable of this reaction. Thiols are similarly good nucleophiles and are known to inhibit the Maillard reaction (Song & Chichester, 1967). Thus, one would expect similarities between the mechanism of reactions of thiols and S(IV) with DH.

The kinetics of the reaction of DH with mercaptoethanol, ME, and glutathione, GSH, resemble those of the DH-S(IV) reaction, but the stoichiometry of the DH-thiol reaction is 1:2 whereas that of the DH-S(IV) reaction is 1:1 (Wedzicha & Edwards, 1991). Aminothiols (cysteine, cysteamine, homocysteine) undergo a fast reaction with DH followed by a subsequent slower release of thiol; this is expected to be a Strecker degradation product. More appropriate anti-browning agents would be amino acid derivatives with a *N*-substituted amino group.

The simplest such cysteine derivative is *N*-acetylcysteine, NAC. This compound is unstable in acidic solution and its less well defined reactivity towards DH is a disadvantage regarding its use as an anti-browning agent (Edwards & Wedzicha, 1992). More suitable derivatives could be dipeptides with a C-terminal cysteine, i.e.



Here we describe the synthesis of cysteine-containing peptides and assess the kinetics of their reaction with DH as well as their anti-browning potential.

EXPERIMENTAL

All chemicals were obtained from Sigma Chemicals or Aldrich. The preparation of dipeptides with a C-terminal cysteine residue was based on the method of Sheehan & Yang (1958). The thiol group of cysteine was protected by conversion to a thiazolidine derivative. Peptides were made from corresponding phthaloylamino acids (glycine, leucine, β-alanine, α-aminobutyric acid, glutamic acid, valine and methionine) by the mixed carbonic anhydride method. After formation of the new peptide, the phthaloyl group was removed by hydrazinolysis and the thiazolidine ring cleaved by treatment with mercuric chloride. The reaction scheme is outlined in Fig. 1. A detailed study of the intermediates in the preparation of glycylcysteine was carried out to check the synthesis, by micro-analysis and ^{1}H NMR.

(i) 4-carboxyl-2,2-dimethylthiazolidine hydrochloride, m.p. 164–166°C (lit. 165–168°C). ^{1}H-NMR ($CDCl_3$/DMSO-D_6): δ1·88 (6H, s, $S(CH_3)_2N$); δ3·60 (2H, d, $CHCH_2S$); Δ4·90 (1H, t, $CH_2CH(COOH)N$); δ10·12 (1H, s, COOH).

(ii) 4-carboxyl-2,2-dimethyl-3-(phthaloylglycyl)thiazolidine. Calc. for $C_{16}H_{16}N_2SO_3.0.5H_2O$: C = 51·2%, H = 5·2%, N = 7·5% S = 8·5%; found: C = 51·0%, H = 5·45%, N = 7·8%, S = 8·65%. ^{1}H-NMR ($CDCl_3$/DMSO-D_6): δ1·88 (6H, s, $SC(CH_3)_2N$); δ3·41 (2H, d, $CHCH_2S$); δ4·48 (2H, s, $COCH_2N$); δ5·11 (1H, t, $CH_2CH(COOH)N$); δ7·84 (4H, m, aromatic); δ9·7S (1H, s, COOH).

(iii) 4-carboxyl-3-glycyl-2,2-dimethylthiazolidine hydrochloride. Calc. for $C_8H_{15}N_2SO_3Cl.H_2O$: C = 35·1%,

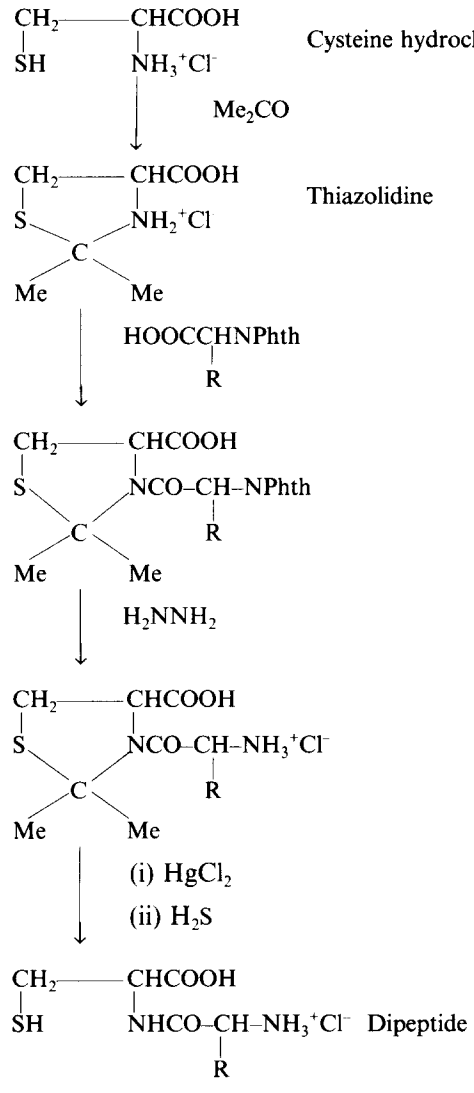


Fig. 1. Reaction scheme for the synthesis of dipeptides with a C-terminal cysteine residue.

H = 6·2%, N = 10·2%, S = 11·7%; found: C = 35·2%, H = 5·75%, N = 10·0%, S = 9·3%. ^{1}H-NMR ($CDCl_3$/DMSO-D_6): δ1·86 (6H, s, $SC(CH_3)_2N$); δ3·37 (2H, d, $CHCH_2S$); δ3·80 (2H, s, $COCH_2N$); δ5·13 (1H, t, $CH_2CH(COOH)N$); δ8·40 (1H, s, COOH).

(iv) Glycylcysteine hydrochloride.
Calc. for $C_5H_{12}N_2SO_3Cl$. (1·4H_2O): C = 24·9%, H = 6·2%, N = 11·6%, S = 13·2%; found: C = 25·4%, H = 6·15%, N = 11·7%, S = 13·75%. ^{1}H-NMR (H_2O): δ3·13 (2H, d, $CHCH_2S$); δ5·40 (1H, t, $CH_2CH(COOH)N$).

The homogeneity of the thiol compounds was established by TLC on silica gel G using butanol:acetic acid:water (4:1:1) as solvent. Thiols were revealed by spraying plates with a solution of 5,5'-dithiobis(2-nitrobenzoic acid), DTNB, in ethanol (0·1% w/v). The yields and R_f values are given in Table 1. The spots corresponding to thiol compounds showed no UV absorption (254 nm) although non-thiol impurities, absorbing in the UV, were present. The R_f values of these impurities in preparations

Table 1. R_f values of the thiol-reactive component of dipeptide preparations, separated by TLC on silica using butanol : acetic acid : water (4 : 1 : 1). Cysteine runs at R_f 0·28

Dipeptide	R_f	Yield (g)
gly-cys	0·55	0·42 (3·5%)[a]
leu-cys	0·66	0·86 (5·0%)
ala-cys	0·55	n.d.[b]
val-cys	0·73	0·93 (7·0%)
aba-cys	0·64	0·60 (4·5%)
glu-cys	0·68	n.d.
met-cys	n.d.	0·28 (2·0%)

[a]Percent yields, given in parenthesis, are based on an initial weight of cysteine hydrochloride of 9·6 g.
[b]n.d., not determined.

of gly-cys, glu-cys and aba-cys were 0·63, 0·81 and 0·90, respectively. When dipeptides were allowed to oxidise by prolonged exposure to air, TLC revealed that the spots which absorbed UV light were disulphides. All dipeptides were obtained in the hydrochloride form as hygroscopic solids and were stored at –20°C. Solutions of peptides were prepared immediately before use and were standardised for thiol content with DTNB reagent (Ellman, 1959).

3-Deoxyhexosulose was prepared by the method of Madson & Feather (1981) and solutions of DH standardised before use as described by Wedzicha & Edwards (1991).

Reaction mixtures containing DH (10–40 mM) and dipeptide (19–40 mM) were prepared in pH 5·5 acetate buffer (50 mM) containing EDTA (0·5 mM) to reduce autoxidation of thiol groups. Reaction mixtures were dispensed into 2-ml vials which were sealed and heated at 55°C. Vials were withdrawn at timed intervals and their contents analysed for thiol. The thiol concentration was determined spectrophotometrically (Ellman, 1959) using DTNB.

Reaction mixtures containing glucose (1 M), glycine (0·5 M) and dipeptide (20 mM), or thiol (ME, NAC, GSH) or S(IV) (20 mM), were prepared in either sodium acetate (0·5 M) or water, and the pH adjusted to 5·5 using acetic acid (1 M) or NaOH (1 M). Reaction mixtures were heated at 55°C, and their absorbances (490 nm) were measured at timed intervals. Aliquots (1 ml) were withdrawn at timed intervals and analysed for thiol content as before. The pH of reaction mixtures was measured at the time of onset of browning.

RESULTS AND DISCUSSION

Anti-browning behaviour of the dipeptides

The effectiveness of dipeptides as anti-browning agents is illustrated in Fig. 2 by their inhibition of browning in unbuffered mixtures of glucose + glycine. The pH of mixtures fell by 1–2 units (time > 400 h) depending upon the thiol used. In general, the fall in pH from an initial value of pH 5·5 was to pH 3·6–4·1 at the onset of browning in the case of the dipeptides, whereas

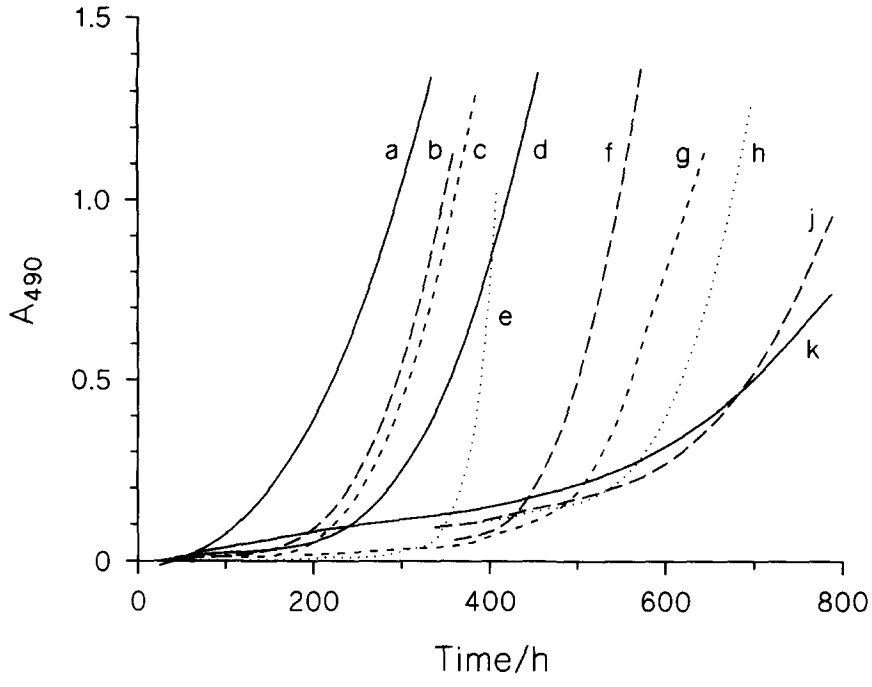


Fig. 2. Absorbance–time curves for the browning of glucose + glycine (a) in the absence of inhibitor and in the presence of (b) gly-cys, (c) val-cys, (d) ala-cys, (e) S(IV), (f) leu-cys, (g) mercaptoethanol, (h) *N*-acetylcysteine, (j) glutathione and (k) cysteine. Reaction conditions: unbuffered, initial pH 5·5, 55°C, [thiol] or [SIV)] = 20 mM, [glucose] = 1·0 M, [glycine] = 0·5 M.

reactions containing other thiols (ME, NAC) and S(IV) had final pH values in the range 4·34–4·55, despite longer reaction times before browning commenced (time > 700 h for the thiols). Mixtures containing cysteine gave rise to cooked-meat aromas, whilst very little smell was evident in reactions containing the dipeptides.

A rapid initial loss of thiol (time < 25 h) was observed in mixtures containing dipeptides, e.g. 54% of leu-cys was bound after 25 h; this was followed by a slow loss of thiol over a much longer time. Glycine (0·5 M) appeared to have no kinetic effect on this reaction; in the absence of glycine there was an initial loss of leu-cys of some 50%, but no browning of the glucose alone took place on the time-scale of the experiments reported here. The extent of the initial loss of dipeptide is similar to that observed in DH-dipeptide reactions; this loss can be explained in the same way by postulating the thiazolidine product which, in the case of glucose, would involve the aldehyde group of the hexose. The significance of the carbonyl group in this reaction is indicated by the fact that pyruvaldehyde and acetaldehyde show similar reactivity; this behaviour is not peculiar to α-dicarbonyl compounds. The slow reaction of the dipeptide, which follows the initial loss, in the glucose–glycine–dipeptide reaction, can be explained here by the gradual formation of intermediates in browning, such as DH, from the glucose+glycine reaction. The reaction of glucose with dipeptide does not account for the anti-browning behaviour of the dipeptides since the glucose concentration far exceeds that of the dipeptides. This is the same situation as encountered when S(IV) is used to inhibit Maillard browning and specific reactions between S(IV) and the intermediates in browning must be invoked.

The inhibitory behaviour of the dipeptides in buffered reaction mixtures is illustrated in Fig. 3. In these cases the pH was stable to better than 0·2 units (time > 200 h). Again, a rapid initial loss of thiol groups (time < 25 h), from glucose–glycine–dipeptide mixtures, was observed, which was followed by a slower loss of thiol. The onset of browning in mixtures containing dipeptides occurred when the thiol concentrations fell below some 10% of their original value.

It is evident that all the dipeptides show similar anti-browning behaviour in buffered media. A significant difference from the near-ideal behaviour of S(IV) is that the dipeptides are not as effective in reducing small amounts of browning in the early stages of reaction. Simple thiols (e.g. ME) are more effective than the dipeptides in reducing browning in the early stages of reaction, but less so than S(IV). The less effective anti-browning behaviour of the dipeptides could be due to an inability of the thiols to effectively scavenge intermediates in browning or the DH-thiol product could be showing significant tendency towards browning.

The dipeptides show different anti-browning behaviour in unbuffered glucose+glycine reactions. A possible explanation is that the different dipeptides exert different kinetic effects on the glucose–glycine–thiol reaction, perhaps by catalysing the early stages of the Maillard reaction in a manner similar to S(IV) (Wedzicha & Vakalis, 1988). Simple thiols are, in fact, know to catalyse the Amadori rearrangement (Ingles, 1963), which is also the point at which it is suggested that S(IV) exerts its kinetic effect. Experiments involving buffered media contained acetic acid+acetate ion, which are know catalysts of browning; the combined effect of the buffer components and the maintaining of a constant pH can be seen by comparing absorbance-time data for buffered and unbuffered browning

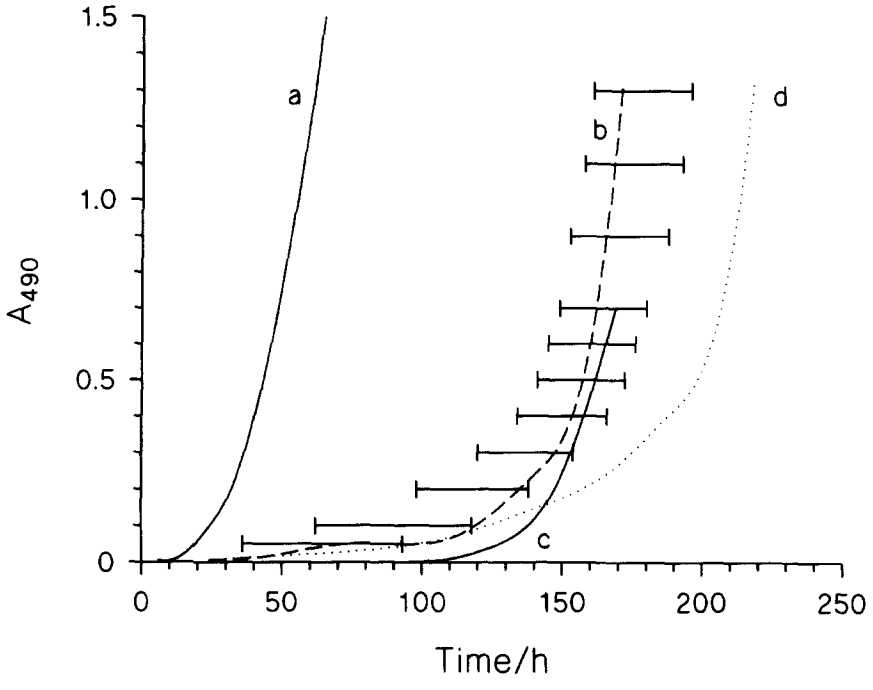


Fig. 3. Absorbance–time curves for the browning of glucose + glycine in buffered solutions, (a) in the absence of inhibitor and in the presence of (b) mercaptoethanol, (c) S(IV), (d) glutathione and the dipeptides. The curves for reactions in the presence of gly-cys, leu-cys, val-cys, aba-cys and glu-cys are within the limits of the horizontal bars. Reaction conditions: 0·5 M acetate buffer, pH 5·5, 55°C, [thiol] or [S(IV)] = 20 mM, [glucose] = 1·0 M, [glycine] = 0·5 M.

reactions, without thiol compound or S(IV), in Figs 2 and 3. The time for browning to begin reduced from 178 to 33 h by the incorporation of the buffer (0·5 M). The possible kinetic effect of the dipeptides in buffered media may be insignificant compared to that of the buffer components, thereby reducing the apparent variation of kinetic behaviour of the individual anti-browning agents.

Reaction of the dipeptides with 3-deoxyhexosulose

The concentration–time characteristics of the reaction of DH with each of the dipeptides are shown in Fig. 4. There appears to be a rapid initial binding of thiol groups, with less than one mole of thiol reacting per mole of DH. A similar stoichiometry has previously been reported (Wedzicha & Edwards, 1991) for the

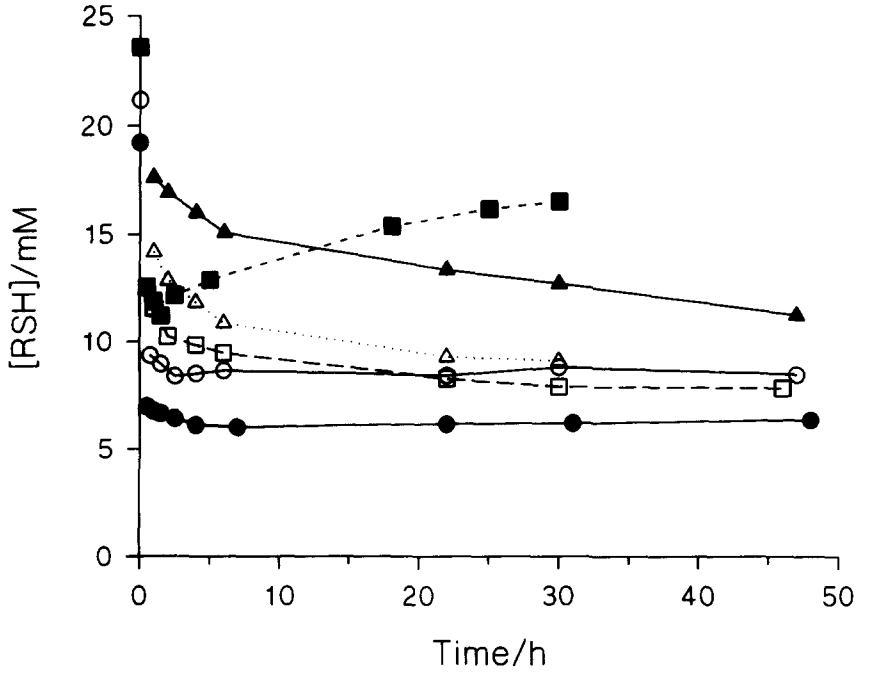


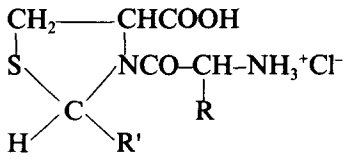
Fig. 4. Thiol concentration–time data for the reaction between dipeptides and DH. Reaction conditions: 50 mM acetate buffer, pH 5·5, 55°C. (○) gly-cys; (□) aba-cys; (Δ) met-cys; (●) leu-cys; (■) ala-cys; (▲) val-cys. The data for glu-cys are superimposable on the curve for val-cys.

reaction between DH and NAC. In the case of ala-cys, the initial binding was followed by a slower partial release of thiol. Glycine (0·5 M) had no effect on the rate or extent of the reaction between DH and gly-cys on the time-scale investigated.

The reproducibility of the results was demonstrated by reacting individual dipeptides from separate preparations of gly-cys and aba-cys, with DH, under identical conditions; concentration–time curves obtained using the different preparations were superimposable and could not be distinguished from one another.

Similar kinetic behaviour was observed when the dicarbonyl compound was pyruvaldehyde (PA). Whilst the reaction of all the peptides with DH showed a rapid initial loss of thiol, the reaction of peptides containing leucine, glycine and β-alanine with PA showed a subsequent slow partial release of thiol. Strecker degradation of simple aminothiols, e.g. cysteine, leads to the formation of low-molecular weight thiols and other sulphur-containing compounds which contribute to a meat-like aroma. Unlike the DH-cysteine reaction (Wedzicha & Edwards, 1991), the initial reaction between dipeptides and DH is not followed by a release of thiol, suggesting that the dipeptides are not degraded, possibly accounting for the lack of a significant aroma in model systems. Whilst a small release of thiol was evident in the reaction between PA and the three dipeptides, this was not accompanied by a significant formation of aroma. A rapid loss of thiol was also observed in mixtures containing acetaldehyde and gly-cys.

A thiazolidine has been proposed as the initial adduct formed between DH and aminothiols (Wedzicha & Edwards, 1991). A similar structure could be envisaged when dipeptides react with the aldehyde group of DH, i.e.



R' = $COCH_2$ $(CHOH)_2CH_2OH$

However, there is no published evidence to suggest that *N*-substituted aminothiols, e.g. NAC, are able to react directly with carbonyl compounds to form such cyclic structures. On the other hand, *N*-substituted thiazolidines do exist and are more stable towards alkaline hydrolysis than those with a hydrogen on the nitrogen atom (Luhowy *et al.*, 1973).

The effect of DH concentration on the thiol concentration–time data in the DH-(gly-cys) reaction is illustrated in Fig. 5. The initial rate was too great for the progress of the initial reaction to be measured using DTNB reagent. Extrapolation of concentration–time data (time >21 h) to zero time allows one to estimate the extent of loss of thiol during the initial rapid reaction phase. At the lowest DH concentration, 5·8 mM, the concentration of thiol which had become unavailable to DTNB reagent was 6·5 mM, and the product, therefore, shows a 1:1·1 (DH:thiol) stoichiometry. However, as the DH concentration is increased, the initial fall in thiol concentration remains approximately the same; the amount of thiol reacted is found to be 6·5, 6·6, 6·8 and 7·0 mmol $litre^{-1}$ at initial DH concentrations of 5·8, 10·1, 15·1 and 21·0 mM, respectively. However, when [DH]≥10 mM, there is a subsequent slow reaction leading to a further loss of thiol. If one assumes that 5·8 mmol $litre^{-1}$ of DH reacts initially with the thiol and the subsequent rate of loss of thiol,

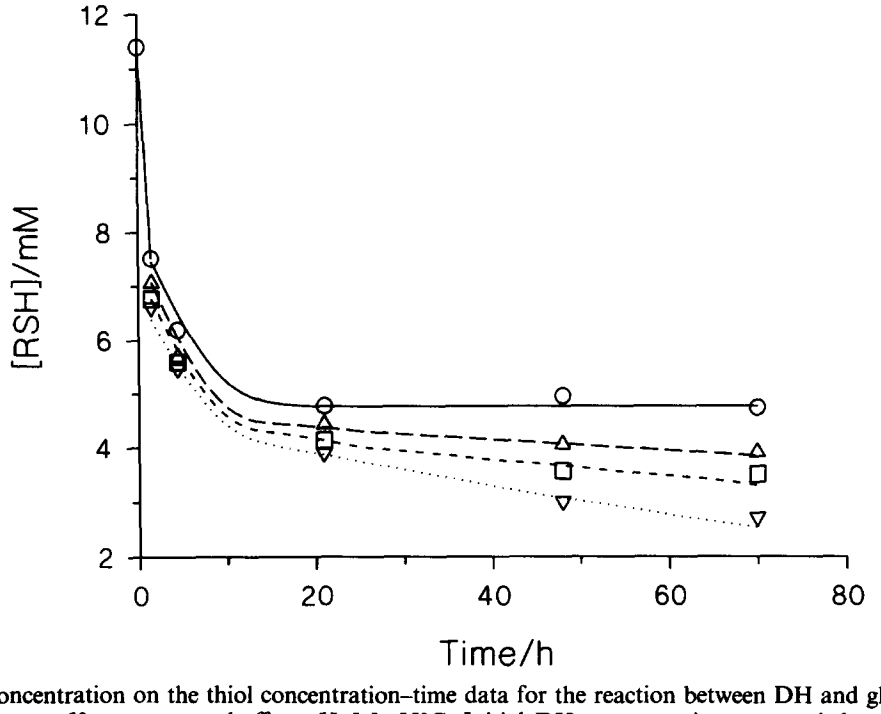


Fig. 5. Effect of DH concentration on the thiol concentration–time data for the reaction between DH and gly-cys. Reaction conditions: [gly-cys] = 11·4 mM; 50 mM acetate buffer, pH 5·5, 55°C. Initial DH concentration was varied as follows: (○) 5·8 mM; (△) 10·1 mM, (□) 15·1 mM; (▽) 21·0 mM.

Table 2. Effect of reactant concentration on the initial rate of the reaction between DH and val-cys. Reaction conditions: 50 mM acetate buffer, pH 5·5, 55°C

[DH] (mM)	[Thiol] (mM)	Initial rate (mM h^{-1})
10·5	25·0	3·17
21·0	25·0	3·65
29·7	25·0	3·52
40·1	25·0	3·49
29·5	40·0	5·18
23·5	19·5	2·31

at different initial DH concentrations, is examined as a function of residual thiol concentration, a linear relationship between rate and concentration is seen, with the line passing through the origin. The reaction of DH with a range of simple thiols has previously been shown also to be of first order with respect to DH (Wedzicha & Edwards, 1991). Similar behaviour was observed for the reaction of DH with val-cys, except that the rate-concentration behaviour was not linear and suggested a order of slightly less than 1 with respect to DH.

Any mechanism for the initial reaction between DH and the dipeptides should reconcile the fact that the *extent* of the initial reaction appears to be independent of DH concentration. The initial rate of reaction between DH and val-cys was sufficiently slow for initial rates to be measured. The effect, on the rate of loss of thiol, of varying the concentrations of both reactants is given in Table 2. The reaction is of zero order with respect to DH and of first order with respect to dipeptide. The rate-determining step of this reaction therefore involves a spontaneous change to the dipeptide molecule which does not require the participation of DH. Any suggestions regarding such a mechanism would be speculative. The relatively constant stoichiometry of the initial DH–dipeptide reaction could, for instance, be the result of a given proportion (approximately half) of the dipeptide being present in a more reactive, but as yet unspecified, form.

CONCLUSION

Dipeptides containing cysteine at the C-terminal end are effective inhibitors of the Maillard reaction of glucose+glycine. The most effective anti-browning dipeptide identified so far is leu-cys which, in terms of the length of time for browning to commence, is more effective than S(IV) at the same concentration. As is known for the behaviour of S(IV), the inhibition of browning by thiols is accompanied by a loss of the inhibitor when measured with DTNB reagent (pH 7–8). In general, the thiol anti-browning agents are not as effective as S(IV) in their inhibition of the formation of small amounts of colour in the early stages of reaction, i.e. whilst there is a high proportion of the inhibitor remaining in the reaction mixture.

The results leave two important questions to be answered. First, it is suggested that the dipeptides might exist in more or less reactive forms; this possibility is currently being investigated by ^{13}C-NMR. Secondly, the chemical fate of potential dipeptide anti-browning agents should be investigated before the use of such compounds in foods can seriously be considered.

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