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Formation of Mutagens in An Amino Acid–Glucose Model System and the Effect of Creatine

Ingolf F. Nes

Norwegian Food Research Institute
PO Box 50, N-1432 Ås-NLH, Norway

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ABSTRACT

Heating of amino acids and glucose in a water solvent system produces substantial amounts of mutagens only at alkaline pH (10·5). Positive response was demonstrated with the Salmonella *tester strains TA 98, TA 1538, TA 97, TA 1537 and TA 100. When creatine was added to this system prior to heating, mutagens were formed at all pHs tested (pH 4, 7, 9 and 10·5) and highest mutagenic activity was recorded at pH 9·0 and pH 7·0. The amount of mutagen produced was dependent on the creatine concentrations. A linear increase of mutagenic activity was recorded in the threonine–glucose system when creatine concentration increased to 0·5*M. *Similar results were also demonstrated in the glycine–glucose system up to 0·2–0·3*M *creatine. The increased amount of mutagenic activity found in the presence of creatine was demonstrated to be active only against the frame shift tester strains (TA 98 and TA 97) and not when tested with the TA 100 strain.*

HPLC analyses indicated that the mutagenic activity found in the pure amino acid glucose system at alkaline pH was also found in the presence of creatine; however, the major peak of mutagenic activity in the creatine containing system was not found in the absence of creatine.

INTRODUCTION

The occurrence of mutagens in food is of various origin. Additives, contaminants, natural constituents and compounds formed during food processing have been shown to possess mutagenicity (for review see Stick, 1982). In particular, heat processing is of main concern with regard to

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mutagen formation in proteinaceous food. However, heat treatment is an inevitable process applied to most food, mainly because of health, dietary and nutritional reasons.

Extensive work has been carried out to quantify and identify mutagens formed from amino acids, proteins and proteinaceous food by pyrolysis involving radical reaction at temperatures above 300°C (Sugimura & Nagao, 1979). Another mode of mutagen formation is the Maillard reaction to yield amino carbonyl compounds followed by Amadori rearrangement and the browning reactions during exposure to low temperature (below 250°C). Throughout the years, numerous investigations have been carried out in model systems, which include reducing sugars like glucose and amino acids, to identify the browning products. Since the discovery of mutagen formation during mild heating of food, the effect of individual constituents of food on the mutagen formation has been studied in model systems. (Yoshida & Okamoto, 1980; Wei *et al.*, 1981; Jägerstad *et al.*, 1983; Shinohara *et al.*, 1983; Spingarn *et al.*, 1983.)

It has been shown that moderate heating of dissolved amino acid and glucose in water forms mutagenic compounds at alkaline pH (Spingarn *et al.*, 1983). When creatine was added to a glycine–glucose mixture dissolved in 86% diethylene glycol, increased formation of mutagens was observed during mild heating (Jägerstad *et al.*, 1983). Later work has identified two mutagens (2-amino-3,7,8-trimethylimidazo[4,5-f]quinoxaline and 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline) from this model system (Negishi *et al.*, 1984; Jägerstad *et al.*, 1984.)

It is of great importance to establish good model systems to investigate the mode of formation of mutagens in heated food. In this paper the mutagenic activity of heated amino acids–glucose–creatine dissolved in water at different pH values was studied.

MATERIALS AND METHODS

Bacterial strains

Salmonella typhimurium strains TA 98, TA 1538, TA 100, TA 1539, TA 97 and TA 1537 were kindly provided by Dr Bruce N. Ames.

Mutagenic assay

The Ames mutagen test was performed as described by Maron & Ames (1983). The S-9 mix contained 5 mg/ml rat liver protein and was isolated from the Aroclor 1254-induced Sprague Dawley rat liver. 200 μl S-9 mix

was added to the preincubation mixture per plate together with 100 μl test bacterium and 100 μl testing substance. Triplicates were always poured for each dose of substance tested. Testing materials were diluted in dimethylsulphoxide (DMSO).

Preparation of browning reaction products for mutagen testing

2·5M solutions of amino acids and glucose were prepared in H_2O.

Solutions of glycine and glucose were mixed in the ratio of 6:1; serine and glucose, and threonine and glucose were mixed in the ratio of 3:1 (Spingarn *et al.*, 1983). The individual mixtures were adjusted to the proper pH by NaOH or HCl and the volumes were adjusted to a final total concentration of both reactants to be 2 M. The heating was carried out for 3 h at 120°C in closed pyrex tubes.

After heating, the mutagenic compounds were extracted three times by 1·5 vol CH_2Cl_2. The dichloromethane was removed from the extracts by evaporation and the material was dissolved in ethanol or dimethylsulphoxide (DMSO). The mutagenic activity found in the original heat-treated mixture was completely recovered by this extraction procedure and resulted in 50–200-fold concentration.

When various amounts of creatine were included in the amino acid–glucose heating mixture, the concentrations of amino acids and glucose were unchanged.

HPLC analyses

Dichloromethane-extracted concentrates of various heated samples of amino acids–glucose–creatine were analyzed by high performance liquid chromatography (HPLC) using a μBondapak C_{18} column (7·8 mm × 30 cm) from Waters.

The column was connected to a Spectra Physics SP 8700 delivery system containing a 50 μl sample injection loop. The detector system was a Hewlett-Packard 1040 HPLC photo diode array detector.

The sample was eluted at a constant flow of 1·5 ml/min starting with 50% methanol and 50% H_2O for 30 min, followed by a linear gradient up to 80% methanol and 20% H_2O for 20 min, and with a linear gradient up to 90% methanol for the last 6 min.

Fractions were collected starting with 6 min fractions, followed by 2 min fractions, in the region of mutagen activity.

The fractions were concentrated by evaporation and dissolved in DMSO (6 min fractions in 450 μl DMSO and 2 min fractions in 150 μl DMSO). 50 μl samples were used in each incubation of Ames mutagen test.

TABLE 1
Mutagenic Activity in Heated Model Systems (Amino Acid/Glucose or Amino Acid/Glucose/Creatine 0·3M) at Different pHs

pH	*Amino acid present in the model system with glucose*	*Revertants per millilitre of reaction mixture*						
		TA 98		*TA 1538*	*TA 100*		*TA 1535*	*TA 97*	*TA 1537*
			+ creatine 0·3M			*+ creatine* 0·3M			
4	Glycine		2080			100			
7	Glycine		3740			160			
7	Glycine		4500			240			
9	Threonine	380		420	200		<5	280	10
9	Glycine	940	1440	1670	380	240	<6	670	67
10·5	Threonine	1920	7400	2800	860	800	<20	1480	100
10·5	Serine	1200		1940	300		<6	500	45

RESULTS

Mutagen activity in heated amino acids and glucose

Glycine, threonine and serine were individually heated with glucose at pH 10·5 and the samples were tested for mutagenic activity with the six *Salmonella* tester strains. The mutagenic activity was assayed with and without S-9 mixture and was shown to be dependent on S-9 activation on all strains tested. Figure 1 shows a typical dose–response experiment where increasing amounts of CH_2Cl_2-extracted mixture of glycine–glucose were added to the *Salmonella* test system. In the absence of S-9 liver extract no significant mutagenic activity was found. When liver extract was present, 5 μl mutagen extracts gave 470 revertants (940 revertants per millilitre reaction volume) with TA 98 tester strain. TA 1538 tester strain gave 1400 revertants per 5 μl extract (2800 revertants per millilitre reaction volume). Part of Table 1 summarizes the mutagenic activity of the heated mixtures of the amino acids and glucose on the six strains tested. The numbers are based on dose–response curves calculated in the linear response area of the curve as shown in Fig. 1.

The heated products were mutagenic to all the test strains except TA 1535. The mutagenic activity was 100% extractable by dichloromethane.

The formation of mutagenic activity was dependent on alkaline pH since

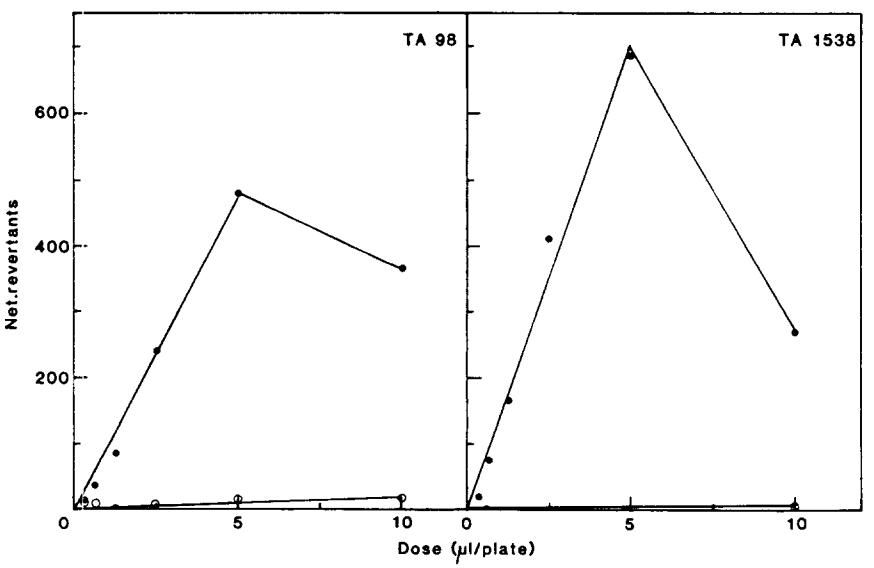

Fig. 1. Mutagenic activity of CH_2Cl_2 extract (50-fold concentrated) of heated glycine–glucose (pH 10·5) tested on TA 98 and TA 1538 *Salmonella* strains. In the presence of S-9 liver extract —●—. In the absence of S-9 liver extracts —○—.

no mutagenic activity was seen when the heating was performed at pH 7 or pH 4 (data not shown). However, at pH 9 some mutagenic activity was observed, particularly in the presence of threonine (Table 1).

Effect of creatine

Under standard reaction conditions, using TA-98, an increasing amount of creatine was added to the threonine–glucose and the glycine–glucose systems at pH 10·5 and pH 9·0, respectively. The results from mutagen testing of the dichloromethane extracts of the heated samples are shown in Fig. 2. In this system the amount of mutagenic activity increases linearly with increasing dose of creatine (Fig. 2A).

The most profound stimulation of mutagen formation was observed in the glycine–glucose system at pH 9·0. While no mutagenic activity was detectable without creatine, a substantial amount of mutagen activity was found even in the presence of the lowest dose of creatine (0·1 M) (Fig. 2B).

When the TA 100 strain was used in the *Salmonella* test system no enhanced mutagenic activity was observed either in the heated threonine–glucose or glycine–glucose samples. In fact, some reduction in mutagen activity was observed with TA 100 strain when 0·3M creatine was added to threonine–glucose (Table 1).

The heating reaction with glycine–glucose in the presence of 0·3M creatine was carried out at four different pH values. The amount of mutagenic activity of the dichloromethane-extracted samples was evaluated by the TA 98 and TA 100 strains, as shown in Table 1.

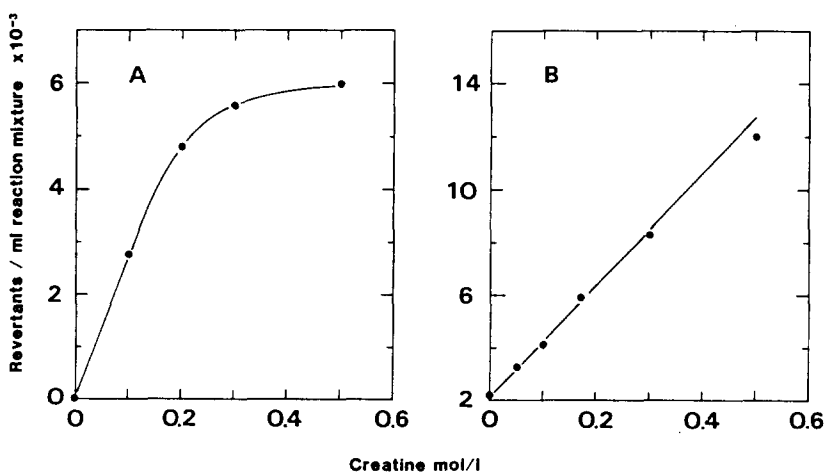

Fig. 2. Mutagenic activity of CH_2Cl_2 extracts of heated model systems containing various amounts of creatine. TA 98 was used as tester strain. A: Glycine–glucose, pH 9. B: Threonine–glucose, pH 10·5.

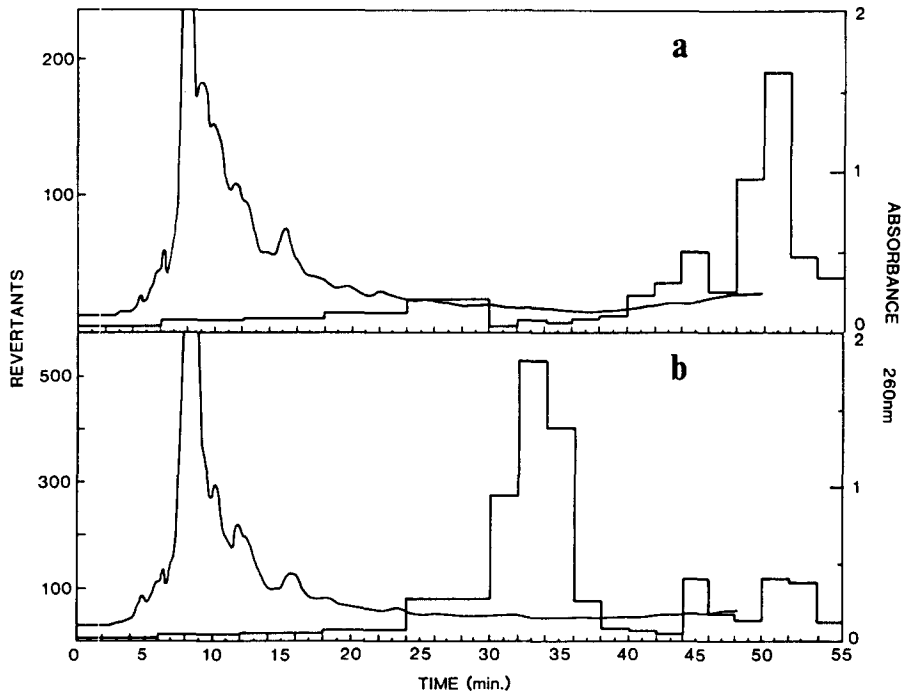

Fig. 3. a, HPLC analysis of CH_2Cl_2 extract and mutagenic activity from heated glucose–threonine (pH 10·5, 150-fold concentrated) on *Salmonella typhimurium* TA 98. b, As 3a, except creatine (0·5M) was included in the heating mixture.

Table 1, as well as Fig. 2, show that creatine has a great impact on the mutagen formation of the amino acid glucose heating systems by enhancing the mutagen formation and by forming the largest amount of mutagens at more neutral pH than what is observed in the pure amino acid glucose systems.

HPLC analyses

The influence of creatine in the mutagen formation could be twofold, either by a stimulatory (catalytic) effect of the yield of mutagens or by being a precursor in forming new mutagens. In a trial to investigate these two possibilities HPLC analyses of dichloromethane-extracted samples of glycine–glucose heated mixtures with various amounts of creatine were carried out. As can be seen from Fig. 3, most of the mutagen activity on *S. typhimurium* TA 98 in the creatine containing sample eluted from the column at a different concentration of methanol (50–60% methanol) compared with the mutagenic activity of the pure amino acid–glucose sample (80–90% methanol). Furthermore, the major mutagen activity

observed in the pure amino acid–glucose sample was apparently found also in the sample containing creatine.

DISCUSSION

The mutagenic activity formed during heating of amino acids and glucose was shown to be pH dependent in a water solvent system.

While a substantial amount of mutagenic activity was formed at pH 10·5 only 15–20% of the activity was found in heating at pH 9·0 (Table 1), and at pH 7 and 4 no mutagenic activity was formed in the threonine–glucose reaction (data not presented). When the heated glycine–glucose was tested, mutagenic activity was recorded only at pH 10·5.

The standard tester strains (TA 97, TA 98 and TA 100), which are recommended for general mutagenesis testing as well as their R-plasmid free strains (TA 1537, TA 1538 and TA 1535), have been used in the present study. The R-plasmid-free TA 1538 was the most sensitive strain in detecting mutagen activity in the heated mixture (Table 1).

In general, the strains (TA 98, TA 97, TA 1538) which are used for detecting frameshift mutagens were more sensitive to mutagens in the heated amino acid–glucose mixture than the strains (TA 100, TA 1535) used in the detection of base-pair substitution. It should also be emphasized that rat liver S-9 extract was an absolute prerequisite for the mutagenicity.

This observation is consistent with previous work which has shown that heated amino acids and glucose at alkaline pH contained significant mutagenic activity on TA 98 and TA 100 with S-9 activation (Spingarn *et al.*, 1983). However, the strong alkaline pH may raise the question of the biological relevance of this model system since alkaline treatments can result in significant changes in the properties of proteins and amino acids. It has been shown that high pH can cause racemization and cross linking of amino acyl residues and, of specific chemical components, lysinoalanine has been shown to be formed (Liardon & Hurrell, 1983). Consequently, it is not unlikely that heating of the herein-described model systems at strong alkaline pH may lead to mutagens different from what is formed at a lower pH.

Several reports have shown that creatine may be an important contributor to mutagenic compounds found in heated food constituent mixtures (Yoshida & Okamoto, 1980; Vithayathil *et al.*, 1983; Yoshida & Fikuhara, 1983). In a model system where amino acids, glucose and creatine were dissolved in 86% dimethylglycol and heated at neutral pH, it has been shown that substantial amounts of mutagens were formed (Jägerstad *et al.*, 1983).

The present water solvent system is also strongly affected by creatine.

The creatine enhances the mutagen formation and changes its pH optimum of formation towards more neutral pH (Table 1). This was observed with the TA 98 tester strain, while creatine did not possess any influence on the mutagenic activity directed against TA 100 (Table 1). The mutagenic compounds which have been identified from moderately cooked foods are far more sensitive to the TA 98 than the TA 100 tester strain, and in that way the creatine–amino acid–glucose seemed to simulate, more effectively, the mutagen formation in meat.

When the dichloromethane extracts from various heated samples were analyzed by reversed phase C-18 column, it was clearly demonstrated that the presence of creatine formed new mutagenic compounds which were not formed in its absence (Fig. 3). While the mutagenic activity from the amino acid–glucose system was eluted at 80–90% methanol, the main components of mutagenic activity in the creatine-containing system were eluted as a broad peak at 50–60% of methanol. It should also be emphasized that the activity, which appeared at 80–90% methanol eluate, was found both with and without creatine. This observation suggested that creatine took part in the formation of mutagens which were not found in the pure amino acid–glucose system. The amount of mutagen formed was also observed to be dependent on the amount of creatine added. In fact, a linear dose–response relationship between creatine and mutagen activity was observed in the threonine–glucose system (Fig. 2). Also, in the glycine–glucose system, a linear response of mutagenic activity was recorded at the initial concentrations of creatine (Fig. 2).

In the study of mutagen formation in model systems consisting of food constituents, it is of great importance to look at the various physical, as well as chemical, parameters. The present work demonstrates clearly how creatine, which is a part of muscle, modulates mutagen formation during heating of amino acids and glucose in a water solvent system.

In order to set up a good model system for studying the molecular mechanisms behind, and the precursors of, the formation of mutagens during heating of food, we feel that one should avoid non-physiological conditions like strong alkaline pH or solvents like diethyleneglycol.

Future work will concern isolation and characterization of the various mutagens formed in the present model system with various amino acids.

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