Food Chemistry **21** (1986) 103–114

Nutritional Quality of Diacetyl/Urea Treated Caseinates

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(Received: 25 November, 1985)

ABSTRACT

Diacetyl markedly improves the heat stability of milk, especially in the presence of urea. The present study was undertaken to investigate the effects of severe heat treatments in the presence of diacetyl and urea on the nutritional value of casein.

Acid casein solutions (7% w/v protein) at pH 6·6 were heated at 120°C for 20 min in the presence of the following: (1) no additive; (2) 10 mM urea; (3) 1·5 mM diacetyl; (4) 5 mM diacetyl; (5) 1·5 mM diacetyl + 10 mM urea; (6) 5 mM diacetyl + 10 mM urea. All caseinates treated with diacetyl or diacetyl plus urea were visibly browned. When compared with caseinate heated alone, available lysine was reduced by 8%, 9·5%, 8% and 7% in the caseinates treated with 10 mM urea, 1·5 mM diacetyl + 10 mM urea, 5 mM diacetyl and 5 mM diacetyl + 10 mM urea, respectively, while treatment with 1·5 mM diacetyl, 5 mM diacetyl and 5 mM diacetyl + 10 mM urea reduced arginine content by 10%, 15% and 11%, respectively, and tryptophan content by 5%, 8% and 8%, respectively. PER, NPR and available methionine were unaffected by diacetyl and/or urea except for a 9% reduction in PER in caseinate treated with 5 mM diacetyl + 10 mM urea.

INTRODUCTION

The remarkably high heat stability of milk, which permits the processing of milk at very high temperatures, is very pH dependent: the heat

Food Chemistry 0308-8146/86/$03·50 © Elsevier Applied Science Publishers Ltd, England, 1986. Printed in Great Britain

stability–pH profile of most milks shows a maximum at ~ pH 6·7 and a minimum at ~ pH 6·9; above pH 6·9 the heat coagulation time increases again (Rose, 1961*a*,*b*). In addition to pH, the heat stability of milk is influenced by several compositional and processing factors; the relevant literature has been reviewed by Fox & Morrissey (1977) and Fox (1982). Although the heat stability of the milk protein system at pH values > 6·5 is very high compared with other protein systems, it may be limiting, especially in concentrated milks.

Shalabi & Fox (1982) showed that several dicarbonyls (glyoxal, substituted glyoxals, diacetyl and 1,2-cyclohexadione) markedly improve the heat stability of milk, especially in the presence of urea. Since diacetyl is an indigenous constituent of fermented dairy products and is a permitted food additive, its potential industrial application for the improvement of the heat stability of milk is obvious. Therefore, it is important to determine whether there are any nutritional consequences arising from treatment of milk with diacetyl. It is known from the work of Yankeelov (1970), Yankeelov *et al.* (1966, 1968) and Riordan (1973) that proteins react with diacetyl and that arginine is the amino acid most susceptible to modification, although lysine and histidine are affected to a lesser extent.

The present study was undertaken to investigate the effects of severe heat treatments in the presence of diacetyl and urea on the nutritional value of casein, the principal protein group in milk. The concentration of indigenous urea in milk is 190–530 mg/litre (~3·2–8·8 mM) (Muir & Sweetsur, 1976; Holt *et al.*, 1978). Fresh milk contains no diacetyl but fermented dairy products contain variable amounts, e.g. butter: 0·25–0·40 ppm (Garcia Villanova & Olea Serrano, 1978) or 2·1 ppm (Fiedler, 1979); cultured buttermilk: 0–8·6 ppm (Wang & Frank, 1981); Cheddar cheese: 0·16–3·35 ppm (Calbert & Price, 1949) although concentrations up to 11·3 ppm were reported in Cheddar cheese containing *Streptococcus diacetylactis* (Keen & Walker, 1974).

MATERIALS AND METHODS

Treatment of caseinate with diacetyl and/or urea

Acid casein was obtained from Kerry Co-Op. Creameries Ltd., Listowel, Co. Kerry. The specifications as supplied by the company were:

maximum moisture, 10%; maximum fat, 1·5%; maximum free acid, 0·2%; ash, 1·2%; protein, 87·1%. A caseinate solution (7%, w/v, protein) was prepared by dispersing 1·6 kg acid casein powder in tap water, adjusting and maintaining the pH at 6·6 with 2M NaOH and bringing to a volume of 20 litres with tap water. After standing at 4 °C for 48 h, the pH was readjusted to 6·6. The caseinate solution was divided into six batches and the following additions made: (1) no additive; (2) urea to a final concentration of 10 mM/litre; (3) diacetyl to 1·5 mM/litre; (4) diacetyl to 5 mM/litre; (5) diacetyl to 1·5 mM/litre and urea to 10 mM/litre; (6) diacetyl to 5 mM/litre and urea to 10 mM/litre. These solutions were placed in 1-litre Erlenmeyer flasks (~600 ml in each) and heated in a laboratory autoclave as follows: 30 minutes' heating to 120 °C, 20 minutes' holding at 120 °C and 30 minutes' cooling to 80–90 °C followed by cooling to room temperature. Solutions were freeze-dried and stored in sealed plastic bags at room temperature.

Animals and diets

Male weanling rats, Wistar strain, were obtained from OLAC (1976) Ltd., Bicester, Great Britain. Upon arrival at our laboratory, the animals were housed in wire-bottomed cages and fed a commercial chow diet for 4 days. The rats were then randomised into eight groups of six rats each (average body weights, 68 ± 3 g) and housed individually in wire-bottomed polypropylene cages for the duration of the experiment under the following environmental conditions: temperature, 22 ± 2 °C; relative humidity, 50 ± 5% with a 12-h light/dark cycle. A continual filtered air-flow ventilation system operated in the animal room. Transient environmental disturbances, including noise, irregular entrances to the room and inconsistent handling of animals, were minimised.

The treated caseinate powders were incorporated into isonitrogenous (10%, w/w, protein), isocaloric (4·0 kcal/g) diets (AOAC, 1975). Two further diets were prepared: a diet based on untreated acid casein powder (10%, w/w, protein) and a protein-free diet in which protein was replaced by sucrose (3%, w/w), dextrin (3%, w/w) and glucose (4%, w/w). Diets and water were fed *ad libitum* to animals for 28 days (protein-free diet for 10 days only) and weight gain and food intake were measured.

Protein efficiency ratio (PER) was determined as the ratio of weight gain (g) to protein intake (g) over a 28-day feeding period (AOAC, 1975). Net protein retention (NPR) was determined as the ratio of the sum of the

mean weight gain (g) of animals fed the test protein and the mean weight gain (g) of the animals fed the protein-free diet to the protein intake (g) for the first 10 days of the feeding period (Bender & Doell, 1957). Apparent digestibility of caseinates was determined as the difference between food N intake (g) and faecal N excretion (g), expressed as a percentage of food N intake (g) over a 7-day period (day 10–day 17 of the feeding period).

Chemical assays

Available lysine in the caseinate powders was determined by the method of Carpenter (1960) and available methionine by the method of McCarthy & Sullivan (1941) following enzymatic hydrolysis by the method of Pieniazek *et al.* (1975*b*).

The tryptophan content of the caseinate samples was determined by the method of Spies & Chambers (1949) and the arginine content by the procedure of Sakaguchi (1925) as modified by Tomlinson & Viswanatha (1974).

The nitrogen contents of the caseinate powders and diets were assayed by the Kjeldahl method (AOAC, 1975) and the protein content estimated as $\%N \times 6{\cdot}38$. The browning index of the caseinate powders was determined by the method of Martinez & Labuza (1968).

RESULTS

Browning index

All caseinate solutions treated with diacetyl or diacetyl/urea were visibly browned after heating while those with no additive or with urea alone showed little or no browning. Caseinates treated with 5 mM diacetyl were more severely browned than those treated with 1·5 mM diacetyl and urea acted synergically with diacetyl (Table 1). Precipitation of browned caseinates from solutions (5 %, w/v) in 0·5M phosphate buffer, pH 7·0, by adjustment of pH to 4·6, yielded clear supernatants, the absorbance of which at 420 nm was negligible. These results indicate that all the brown pigment was associated with the protein.

NPR values, PER values and apparent digestibility of treated caseinates

Heat treatment of the caseinate (diet H) caused a 12 % reduction ($p < 0{\cdot}05$) in NPR in comparison with acid casein. Caseinates treated with

TABLE 1
Browning Index Values for Treated Caseinates

Treatment[a]	*Browning index* ($A_{420} \times 100$)
Acid casein	0
H	2·5
H–U	6·5
H–1·5D	33
H–U–1·5D	40
H–5D	58
H–U–5D	63

[a] H: heated (120°C × 20 min) caseinate (no additive); H–U: caseinate heated with 10 mM urea; H–1·5D: caseinate heated with 1·5 mM diacetyl; H–U–1·5D: caseinate heated with 10 mM urea and 1·5 mM diacetyl; H–5D: caseinate heated with 5 mM diacetyl; H–U–5D: caseinate heated with 10 mM urea and 5 mM diacetyl.

diacetyl and/or urea (diets H–U, H–1·5D, H–U–1·5D, H–5D, H–U–5D) had similar NPR values which did not differ significantly from the NPR of heated caseinate (H) (Table 2).

The PER of heated caseinate (H) was 4 % lower than that of acid casein but this reduction was not statistically significant ($p = 0{\cdot}07$). Treatment of caseinate with 5 mM diacetyl and 10 mM urea (diet H–U–5D) significantly ($p < 0{\cdot}01$) reduced PER by 9 % compared with heated casein (diet H). The PER values for all the other caseinates treated with diacetyl and/or urea (diets H–U, H–1·5D, H–U–1·5D, H–5D) were not significantly different from that of heated caseinate (H) (Table 2).

Apparent digestibilities of all caseinates were high and apparent digestibility was not significantly affected by heat treatment alone or by heating with diacetyl and/or urea (Table 2).

Amino acids content of treated caseinates

Heat treatment of caseinate (H) resulted in a significant ($p < 0{\cdot}001$) reduction (10 %) in available lysine (Table 3). When compared with heated caseinate (H), available lysine was further reduced significantly ($p < 0{\cdot}01$) by 8 %, 9·5 % 8 % and 7 % in caseinates treated with 10 mM urea (H–U), 1·5 mM diacetyl plus 10 mM urea (H–U–1·5D), 5 mM diacetyl (H–5D) and 5 mM diacetyl plus 10 mM urea (H–U–5D), respectively. The

TABLE 2
NPR Values, PER Values and Apparent Digestibilities of Treated Caseinates

Diet[a]	*PER*	*NPR*	*Apparent digestibility (%)*
Acid casein	2·73 ± 0·10	3·51* ± 0·24	97·8 ± 0·9
H	2·61 ± 0·09	3·09 ± 0·35	98·3 ± 0·4
H–U	2·67 ± 0·14	3·27 ± 0·58	98·6 ± 0·1
H–1·5D	2·58 ± 0·08	2·94 ± 0·29	98·2 ± 0·3
H–U–1·5D	2·66 ± 0·15	3·21 ± 0·32	98·1 ± 0·4
H–5D	2·63 ± 0·10	3·21 ± 0·20	97·9 ± 0·4
H–U–5D	2·37* ± 0·13	2·80 ± 0·40	98·2 ± 0·2

[a] H: heated (120°C × 20 min) caseinate (no additive); H–U: caseinate heated with 10 mM urea; H–1·5D: caseinate heated with 1·5 mM diacetyl; H–U–1·5D: caseinate heated with 10 mM urea and 1·5 mM diacetyl; H–5D: caseinate heated with 5 mM diacetyl; H–U–5D: caseinate heated with 10 mM urea and 5 mM diacetyl.
* Denotes values which differed significantly ($p < 0.05$) from heat-treated caseinate (H).

TABLE 3
Available Lysine, Available Methionine, Tryptophan and Arginine Contents of Caseinate Preparations[a]

Treatment[b]	*Available lysine (g/16 g N)*	*Available methionine (g/16 g N)*	*Tryptophan (g/16 g N)*	*Arginine (g/16 g N)*
Acid casein	7·99* ± 0·02	2·96 ± 0·01	1·31* ± 0·04	4·75* ± 0·06
H	7·20 ± 0·19	2·92 ± 0·02	1·25 ± 0·01	4·61 ± 0·01
H–U	6·65* ± 0·17	2·96 ± 0·07	1·28 ± 0·01	4·81* ± 0·01
H–1·5D	7·32 ± 0·06	2·75 ± 0·01	1·19* ± 0·02	4·17* ± 0·04
H–U–1·5D	6·52* ± 0·16	2·97 ± 0·09	1·29 ± 0·05	4·64 ± 0·07
H–5D	6·62* ± 0·25	2·81 ± 0·05	1·15* ± 0·03	3·94* ± 0·23
H–U–5D	6·74* ± 0·03	2·83 ± 0·02	1·15* ± 0·04	4·10* ± 0·15

[a] H: heated (120°C × 20 min) caseinate (no additive); H–U: caseinate treated with 10 mM urea; H–1·5D: caseinate heated with 1·5 mM diacetyl; H–U–1·5D: caseinate heated with 10 mM urea and 1·5 mM diacetyl; H–5D: caseinate heated with 5 mM diacetyl; H–U–5D: caseinate heated with 10 mM urea and 5 mM diacetyl.
[b] Values presented as means ± one standard deviation, based on the results of four replicate analyses.
* Denotes values which differed significantly ($p < 0.01$) from those for heat-treated caseinate (H).

available lysine content of caseinate heat treated with 1·5 mM diacetyl (H–1·5D) was not significantly reduced on comparison with heated caseinate (H).

The available methionine content of caseinate was not affected by heat treatment alone or heat treatment in the presence of diacetyl and/or urea.

Heat treatment of caseinate (H) resulted in a significant ($p < 0{\cdot}01$) reduction (5 %) in tryptophan content compared with acid casein. Heat treatment of caseinate with 1·5 mM diacetyl (H–1·5D), 5 mM diacetyl (H–5D), or 5 mM diacetyl plus 10 mM urea (H–U–5D) resulted in further significant ($p < 0{\cdot}005$) reductions of 5 %, 8 % and 8 %, respectively, in comparison with heated caseinate (H). There was no significant difference between the tryptophan content of heated caseinate (H) and those caseinates treated with 10 mM urea (H–U) or 1·5 mM diacetyl plus 10 mM urea (H–U–1·5D).

Heat treatment of caseinate (H) caused a slight reduction in arginine content. Heat treatment of caseinate with 1·5 mM diacetyl (H–1·5D), 5 mM diacetyl (H–5D) or 5 mM diacetyl plus 10 mM urea (H–U–5D) resulted in reductions of 10 %, 15 % and 11 %, respectively, when compared with the heated caseinate (H). The arginine content of caseinate treated with 1·5 mM diacetyl plus 10 mM urea (H–U–1·5D) was similar to that of the heated caseinate (H). Addition of 10 mM urea to caseinate (H–U) resulted in an apparent 5 % increase in the arginine content in comparison with the heated caseinate (H).

DISCUSSION

The reduction of 10 % in available lysine on heating caseinate at 120 °C for 20 min may be due to reaction of the ε-NH_2 of lysine with the amide groups of asparagine and glutamine, with the reacting units either in the same peptide chain or in neighbouring ones. Such reactions have been reported (Bjarnason & Carpenter, 1970) to occur on heating other carbohydrate-free proteins, e.g. bovine plasma albumin at 115 °C. A further reduction (8 %) in available lysine occurred when diacetyl was included at a concentration of 5 mM during heating but not when it was included at 1·5 mM. The loss of available lysine at the higher diacetyl concentration is probably due to reaction of the ε-NH_2 group of lysine with diacetyl, a reaction which has been reported (Yankeelov, 1970) to occur on exposure of protein (bovine serum albumin) to diacetyl at 25 °C at pH 6·0 and 8·2.

Inclusion of urea at a concentration of 10 mM during heating (with or without 1·5 mM diacetyl) also reduced available lysine by 7–9·5% compared with heated caseinate (H). Urea has been reported to react with lysine in proteins to form homocitrulline (Means & Feeney, 1971; Takahashi, 1977).

The tryptophan content of caseinate was reduced by 5% on heating. Much greater losses of protein tryptophan have been reported to occur on heating as skim milk powder containing 2·45% moisture at 120°C and the half-life for tryptophan destruction was estimated to be 18 min under these conditions (Dworschak & Hegedus, 1974). In the present study, the tryptophan losses were probably a result of oxidation of the indole ring with substitution of oxygen at the second position (Sundberg, 1970). This substitution renders tryptophan unreactive to *p*-dimethylamino-benzaldehyde in the Spies–Chambers reaction (Friedman & Finley, 1971).

Inclusion of 1·5 and 5 mM diacetyl with caseinate during heating caused further tryptophan reduction of 5% and 8%, respectively. Yang & Schwert (1972) reported that free tryptophan does not react with diacetyl at 25°C. However, tryptophan decomposition occurs at a much faster rate in skim milk powder at 2·45% moisture heated at 120°C than in dried egg heated under similar conditions, suggesting that lactose may accelerate tryptophan decomposition at high temperatures (Dworschak & Hegedus, 1974). The mechanism by which this occurs is unknown but it is possible that diacetyl may act in a manner similar to lactose at high temperatures. Inclusion of 10 mM urea with caseinate seemed to protect tryptophan against losses caused by heating and by heating in the presence of 1·5 mM diacetyl but not in the presence of 5 mM diacetyl.

Although only a small reduction (~3%) in the arginine content of caseinate occurred on heating, inclusion of diacetyl at 1·5 and 5 mM during heating resulted in further arginine losses of 10% and 15%, respectively. Diacetyl has been reported to react with arginine residues in proteins with a high degree of specificity at pH 6·0, resulting in a reddish-brown discoloration (Yankeelov *et al.*, 1966, 1968; Riordan, 1973) and reaction of bovine serum albumin with 0·4 M diacetyl at 25°C for 48 h reduced arginine content by 75% at pH 6·0 and 93% at pH 8·2 (Yankeelov, 1970). The arginine content of caseinate was increased slightly by the inclusion of 10 mM urea during heating, with or without diacetyl. This apparent increase may be due to the formation of homocitrulline by reaction of urea with lysine in these systems.

Homocitrulline, which is a structural analogue of arginine, may interfere with the measurement of arginine by the Sakaguchi assay. Only negligible losses of available methionine occurred as a result of heating caseinates alone or in the presence of diacetyl and/or urea.

While there was a significant decrease (~ 12 %) in the NPR of caseinate on heating, the decrease in PER (~4 %) did not quite attain statistical significance. In general, inclusion of diacetyl and/or urea did not cause further decreases in PER or NPR, with the exception of caseinate treated with both 10 mM urea and 5 mM diacetyl (H–U–5D), for which the PER was significantly reduced by 9 % compared with heated caseinate (H). These losses in protein quality, as determined by rat growth assays, cannot be readily explained from the changes in amino acids contents of these caseinates.

Thus, the observed losses in lysine, arginine and tryptophan are inadequate in themselves to make them more limiting than the sulfur-amino acids and cannot, therefore, directly account for the observed losses in PER and NPR. Reduced availability of the sulfur-amino acids, particularly methionine which constitutes ~ 89 % of the total sulfur-amino acid content of casein, would appear to be the most likely cause of the observed reduction in PER and NPR. However, only negligible losses of available methionine were observed with the assay used. The assay for available methionine is based on *in vitro* proteolytic digestion of protein followed by precipitation of undigested protein with $HClO_4$ and measurement of methionine in the supernatant (Pieniazek *et al.*, 1975*b*). From a careful examination of published results (Pieniazek *et al.*, 1975*a*,*b*), it appears that this assay for available methionine underestimates the reduction in protein quality of heat-treated proteins as measured using biological or growth assays on rats.

Available methionine in proteins can be reduced by heat treatment in two ways: (1) heat treatment can cause oxidation of methionine to methionine sulfone which is not utilized by the rat (Miller & Samuel, 1968, 1970) or to methionine sulfoxide which is only partially utilized by the rat (Ellinger & Palmer, 1969); (2) by reduction of protein digestibility due to modification of methionine and/or other amino acids (Cuq *et al.*, 1973; Donoso *et al.*, 1962; Bjarnason & Carpenter, 1970). It is possible that reduced overall digestibility of casein may have been the cause of the observed reductions in PER and NPR. The observation that the apparent digestibility of caseinates was not affected by any of the treatments used, despite losses in lysine, arginine and tryptophan, suggests that apparent

digestibility is not a good measure of true digestibility. This is borne out by other studies (Henry & Kon, 1950; Donoso *et al.*, 1962; Miller *et al.*, 1965) and it has been suggested that incompletely digested fragments of heat-damaged proteins may be absorbed from the small intestine and excreted in the urine (Ford & Shorrock, 1971; Hurel & Carpenter, 1977). Such a mechanism would account for the reduction in PER and NPR observed in this study despite lack of change in apparent digestibility.

In conclusion, it can be stated that addition of diacetyl and/or urea to caseinate before heat treatment at 120 °C for 20 min did not greatly affect protein quality over and above the changes in protein quality attributable to heat treatment alone.

ACKNOWLEDGEMENT

Financial support from EEC Co-Responsibility Fund, Reg. 271/82, is gratefully acknowledged.

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