

Bitter Compounds from Cheddar Cheese

J. EDWARDS¹ and F. V. KOSIKOWSKI

Department of Food Science

Cornell University

Ithaca, NY 14853

ABSTRACT

Bitter peptides were isolated from Cheddar cheeses rendered bitter by milk coagulating agents and from nonbitter cheese. More than one bitter peptide apparently was produced by each coagulant. The peptides generally differed in such properties as elution volume from Sephadex gel, migration on etha gel, and relative amino acid composition. Bitter peptides contained relatively large amounts of aliphatic, acidic, and hydroxy amino acids but small amounts of basic and aromatic acids. The most consistent factors were relatively large amounts of glutamic acid, proline, leucine, and valine and the ratio of aliphatic to acidic amino acids (8 to 1.3).

INTRODUCTION

Bitter flavor of cheese is a common phenomenon, which has stimulated considerable inquiry on causative factors (2). Cheese prepared with rennet or rennet substitutes may become mildly or intensely bitter without apparent reasons. However, the specific nature and amount of milk coagulating agents, type of starter, and pH generally are considered to be contributing factors (3, 5, 12-13, 14).

Knowledge is evolving steadily on components of bitter flavor in cheese. Certain peptides have been implicated, whereas the role of free amino acids remains more obscure. Rasmussen (15) isolated water-soluble bitter peptides from bitter and nonbitter Gouda cheeses. Waudry (20) in 1955 isolated bitter peptides from bitter Cheddar cheeses made with a fungal enzyme, papain, and rennet. Later Harwalker and Elliott (4, 6) extracted bitter Cheddar cheese with chloroform and methanol and obtained an

extremely bitter fraction. Hamilton et al. (3), Rasmussen and Creamer (16), Schalmans and Beunke (17), and Sullivan and Jago (19) also investigated roles of bitter peptides in cheese flavor.

In attempts to isolate the bitter components of cheese, Heringa and Parmela (7) and Harwalker and Elliott (6) extracted components from bitter cheese and fractionated them with Sephadex G-25.

Objectives of our study were to isolate and characterize bitter peptides from aged bitter Cheddar cheese and note relationships between bitter flavor intensity, peptide type, and amino acids.

MATERIALS AND METHODS

Cheddar cheese was prepared by the method similar to that described by Kosikowski (10) with pasteurized milk, but the pH was neutralized a step more than normal to activate enzymes. In the experimental series all rennet, fungal milk coagulating enzymes, and fish were used at equivalent coagulating power along with 1% of active commercial lactic starter. Commercial fungal milk coagulating preparations consisted of three from *Endothia parasitica* (Lander 1 to 3), one from *Alcaligenes eutrophus* (Lander 4), and one from *Alcaligenes eutrophus* (Lander 5).

The resulting blocks of Cheddar cheese after overnight pressing in 10 kg forms were dried on shelves for 24 h and cut into approximately .5-kg rectangular pieces, vacuum sealed in aluminum-polyethylene pouches, and held at -20°C. Cheeses were evaluated over 20 mo for bitterness on a bitterness scale from 0 to 5 (none to extremely bitter). After 1 mo, when strong bitterness had developed, 5 kg of each cheese were frozen at -20°C. Bitter fractions were isolated from these cheeses after thawing.

Preparation of Bitter Extracts from Cheese

The water extraction procedure is dis-

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²To whom all correspondence should be sent.

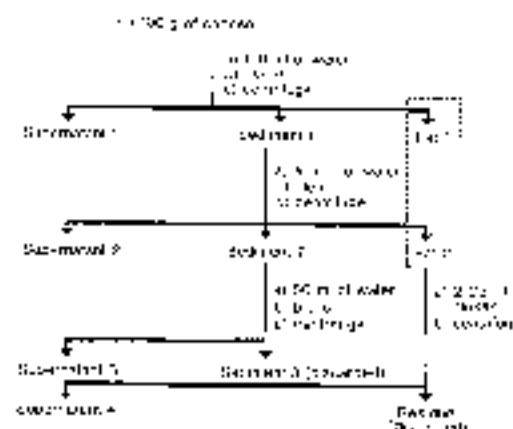


Figure 1. Water extraction procedure for butter extracts from cheese.

grammer (Figure 1). Blending consisted of exposing cheese samples to 2 to 15-s intervals at high speed in a Waring Blender. Mixtures were centrifuged about 10 min at $3,000 \times g$ and fractions separated. Fat portions were combined, rinsed two times with warm water, and rinsings decanted, combined, and centrifuged. Protein sediment was washed two times after initial separation. Protein and fat residues were discarded after final wash, and combined supernatant fractions were stored at 4°C .

The supernatant was concentrated under vacuum at 15°C by a rotary evaporator. Each batch operation consisted of concentrating about 650 ml of supernatant in a 2-liter flask to a thick paste.

The thick paste from each 650 ml of supernatant was extracted with 4- to 50-ml portions plus 2- to 25-ml portions of 95% ethanol. Combined extracts were centrifuged for about 5 min at $10,500 \times g$, and the supernatant was decanted. The residue was discarded after washing with 50 ml of 95% ethanol, centrifuging, and combining the supernatant with the original supernatant. A heat evaporator removed the ethanol from the extract.

The residue after ethanol removal was washed out of the flask with 10 or 20-ml portions of distilled water until a total of 40 ml was added per 650 ml of initial water supernatant. This extract was dialyzed for 2 h against cold, distilled water. The water (about two times the volume of initial water supernatant) was changed after 1 h.

The dialyzed extract was centrifuged about 5 min at $16,500 \times g$. A fragile top sediment was observed usually without bottom deposit. Small amounts of the top sediment decanting with the supernatant were removed by filtering through coarse porosity sintered glass. The extract was stored at 4°C until used for Sephadex gel filtration.

Sephadex Gel Filtration

Original separation of the components in the butter extract was obtained with gels from Sephadex G-25 fine (Pharmacia Fine Chemicals Inc.). Two Kenex Chromatex columns (2.5×100 cm) equipped with a fine porosity Chromatex glass disc were prepared with a gel height of 90 cm.

For each coagulant except lactin, 20 ml of samples was separated in each experimental trial with a flow rate of approximately 1 ml per h. Sample size or flow rate reduction did not improve separation significantly. However, better separation was obtained with 10 ml of the lactin extract as an extremely broad initial peak was eluted when 20 ml was used.

The elution buffer was 0.1 N ammonium hydroxide prepared with redistilled (glass) water. Fractions were collected automatically in 10-ml fractions with the eluant being monitored continuously by a recording ultraviolet analyzer at 254 m μ through a 5-mm path.

Collected fractions were stored at 4°C until examined. Equivalent fractions then were combined according to the ultraviolet elution pattern. Each fraction was dried under vacuum at 45°C in the rotary evaporator and transferred to a storage vial.

Thin Layer Chromatography (TLC)

The solvent system was n-propanol, acetic acid, and water (5:1:3). Silica gel N was chosen for isolation trials because it resulted in good separation, contained no binder, and was almost frag-free in the eluting agent (5% acetic acid). Separation was effected with 250 μ layer of silica gel N on 20 $\times$ 20-cm glass plates.

Sixteen plates were used for each sample in the isolation trials. The gel on each plate was scored 1 cm from each side and 1.5 cm from the top. An 18-cm streak of sample (80 to 90 μ l of 200 mg/ml) was applied 2.5 cm above the bottom of each plate as a series of small spots.

Plates were developed at room temperature in chromatographic jars until the solvent front was arrested by the line scored across the top.

After air drying overnight, the plates were observed under ultraviolet light (Chromatovac, Model CC-20, Chromatovler Products, Inc.), and the fluorescent areas were marked. Two plates were stained by spraying with 1% ninhydrin in acetone. Heating about 5 min at 100°C increased the staining intensity, and handling was facilitated by spraying the stained plates with an acrylic resin (Shimadzu, Nutritional Biochemicals Corporation).

The relationship between fluorescence and ninhydrin reaction was determined and used as a guide in collecting the bands from the unsprayed plates. Fractions were obtained with a vacuum sample collector, impinging the gel plate surface in 5% acetic acid.

Each sample was eluted from the gel with portions (10 ml each) of 5% acetic acid by the Kienbo chromatogramming columns. The gel after elution was tested for biuret and ninhydrin reactions, and eluted fractions were dried under vacuum at 45°C and dissolved in 2 portions (2 ml each) of distilled water.

Analysis of Fractions

For analysis of the Sephadex fractions, solutions containing 10 µg amino acid were prepared. The TLC isolates were tested by solutions prepared after isolation. Tests were for ester, pH, biuret, ninhydrin, ninhydrin, and

ammonia. Amino acid analyses were on acid hydrolysates of the more bitter TLC isolates with an automatic amino acid analyzer (Model 120 C, Spinco Division, Beckman Instruments Inc.). Tyrosine was analyzed when only one peak eluted in the region of tyrosine and phenylalanine in an amino acid analysis. Fractions in sealed, evacuated test tubes were hydrolyzed in 6 N hydrochloric acid for 22 h at 110°C. Excess hydrochloric acid was removed by vacuum evaporation over solid sodium hydroxide. A dilution of the hydrolysate was prepared to contain about 1 µmole of ninhydrin-positive material (as leucine)/ml. Approximately 300 to 500 µl of dilute hydrolysate were analyzed with each column. The amount (µmole) of each amino acid was calculated from the graph by the height-times-width method of Speckmann et al. (18).

RESULTS

Bitterness of Cheeses

Bitterness scores for the cheeses are in Table 1. Relative bitterness (most to least) was firm, endothia-1, endothia-2, rennet or endothia-3, mucor-2, and amcor-1. Bitterness, stronger in the better cheeses after 4 wk at 10°C, remained strong throughout the 20 wk. Cheeses of 4 wk at 10°C were extracted for the bitter fraction as continuous boiling only would produce more water-soluble components, thus complicating the isolation procedure.

TABLE 1. Bitterness scores^a of aging cheeses made with various coagulants.

Coagulum	Weeks at 10°C								
	0 ^b	1	2	3	4	5	8	12	20
Rennet	0	0	1.5	2.5	3.5	4.5	5.5	6.5	7
Endothia-1	3	3.5	4	4.5	5	5	5	4.5	4.5
Endothia-2	0	2.5	3	—	—	5	4	4	3.5
Endothia-3	1.5	1	2	3	3	3.5	3	3.5	3.5
Mucor-1	0	0	0	0	0	.5	0	0	0
Mucor-2	0	0	0	0	1	1.5	1	0	1.5
Amcor-1	1	3	2	5	2	—	—	—	—

^a Bitterness scores: 0 to 5 (less to extremely bitter).

^b After pressing.

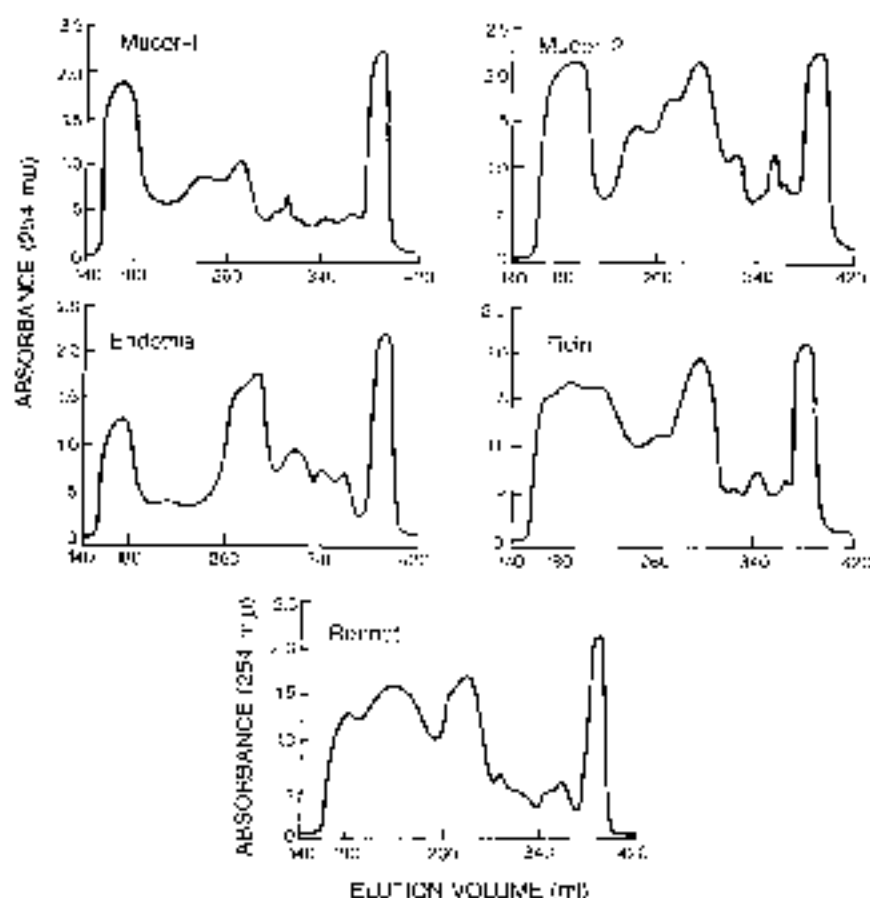


Figure 2. Representative elution patterns from Sephadex G-60 of bitter extracts from cheeses made with coagulants indicated.

Isolation and Characterization of the Bitter Components

Sephadex Gel Filtration. A characteristic elution pattern for each coagulant was obtained by Sephadex gel filtration of the bitter components extracted from each cheese (Figure 2). The first and last peak on each narrow, flow-over-eluted in the same volume ranges.

A comparison of elution volumes of fractions and peaks $\pm$ maximum height and of bitterness scores of fractions obtained with each enzyme is shown (Figure 3). Maximum bitterness eluted from 260 to 300 ml for mucorin 1 to 3, 215 to 280 ml for mucorin 2, 200 to 260 ml for mucorin 3, and 180 to 245 for rennet, indicating the relative order of size (smallest to largest) of components of maximum bitterness

from each coagulant. Only slight bitterness seemed to occur with rennet in the elution range of maximum bitterness for endothia 1 to 3. The strong bitterness of ficin eluted over a wide range (160 to 310 ml) with apparent maxima from 205 to 240 ml and 275 to 310 ml indicating a wide range in size of bitter components produced by ficin.

Fractions eluting before 300 ml showed a positive biuret test, the next fraction was questionable, and the remaining fractions were negative. All bitter-tasting fractions were biuret positive, and acid hydrolysis of the bitter fractions removed all bitterness. Thus, the bitterness produced in cheese by coagulants appeared to be from one or more peptides.

Tests of solutions containing 10 mg/ml of

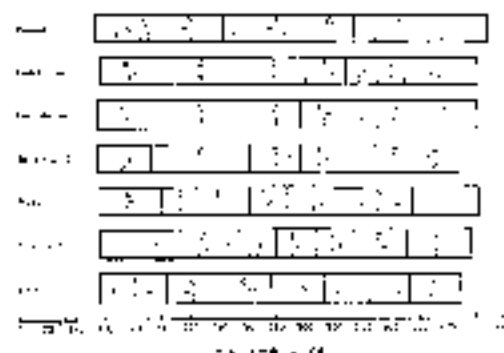


Figure 3. Average elution volume from Sephadex G 25 of the bitter extracts from cheeses made with the various coagulants. Letter = fraction code, number = bitterness score, and 0 = average peak elution volume at maximum height.

dry solids from each fraction indicated no consistent relationship between bitterness and pH, carbohydrate content, or cytophosphat content.

High Resolution by Thin Layer Chromatography. The TLC patterns of bitter Sephadex fractions and additionally areas collected at volume are bitter components along with the bitterness scores are in Figure 4. Different patterns with 10 to 12 ninhydrin positive bands were observed for each type of coagulant. The R_f (not presented) varied, but each TLC pattern was reproducible.

The more bitter fractions of rennet, endocoin 1 to 3, and finin migrated about the same distance on TLC with those of rennet and finin traveling slightly further than those of endocoin 1 to 3. Migration of the more bitter areas of mucor-1 and mucor-2 differed from each other as well as from the other enzymes. Bitter reactions of fractions using letter were positive. A few fractions showing only slight bitterness (.5) were questionable. Trypanine reaction or pH of the fractions was unrelated to bitterness.

Amino Acid Analysis. After acid hydrolysis, an amino acid analysis was made on the more bitter TLC isolates from each coagulant except finin. The results are relative to glycine (Table 2). All of the highly purified isolates contained relatively large amounts of glutamic acid and proline, and most were high in leucine. The amounts of serine and threonine often were about one-half that of glycine. Generally, histidine, tyrosine, and methionine were low.

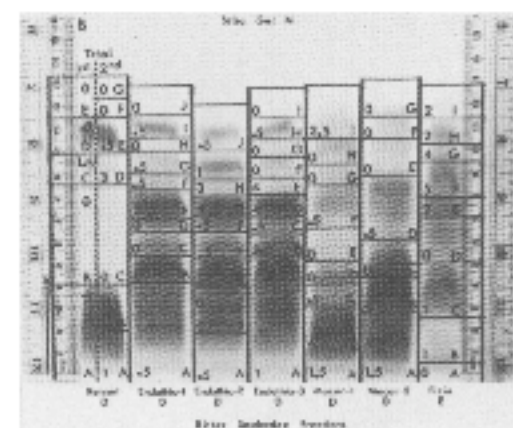
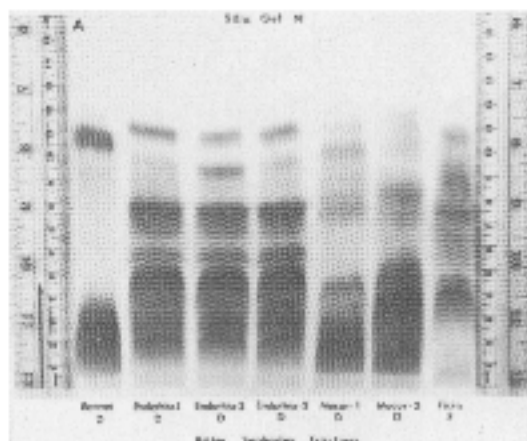


Figure 4. Thin layer chromatogram of bitter Sephadex isolates. In part B, numbers = bitterness scores and letters = fraction codes.

Amino acids were totaled in their respective groups of acidic, basic, aliphatic, aromatic, and hydroxy (Table 3). Most fractions were low in basic and aromatic amino acids but high in acidic, aliphatic, and hydroxy amino acids. The mucor fractions, however, were relatively high in basic amino acids. The most consistent ratio was that of aliphatic to acidic amino acids, which ranged from .8 to 1.3. From aromatic, an amide content of 40 to 60% usually was indicated.

DISCUSSION

Peptides and free amino acids of bitter cheese and their roles and interrelationships represent much current research as illustrated by Kozys et al. (11), who correlated a series

TABLE 2. Amino acid and ammonia content of butyric than after chromatography, isolates expressed relative to glycine equal to one.

Amino acid	Concentrations												
	R			R			R			R			
	0.25			1.0			5.0			50.0			
	Fraction												
S, liters/g. M													
A	C	D	B	D	B	A	C	D	B	A	C	D	
Asp	1.8	1.5	4.8	4	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	
Pro	1.7	1.1	1.3	0	1	1	1	1	1	1	1	1	
His	4.1	3.0	6.6	7.8	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	
Arg	1.1	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2	
Asp	4	8	1.4	1.5	1.1	1.5	1.5	1.5	1.5	1.5	1.5	1.5	
Val	2	3	1.0	1.4	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	
Ser	3	6	1.0	6	1	1	1	1	1	1	1	1	
Glu	3.5	3.0	0.1	3.9	1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.6	
Pro	1.7	1.1	1.4	2.8	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	
Gly	1.9	1.0	1.0	1.1	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	
Ala	1.3	1.8	1.6	1.7	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1	
Val	1.4	2.1	1.4	2.0	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1	
Met	1.2	1.1	1.7	1.1	1	1	1	1	1	1	1	1	
Ileu	1.0	1.5	1.6	1.6	1.7	1.0	1.0	1.0	1.0	1.0	1.0	1.0	
Leu	2.5	1.6	2.0	1.0	1.9	1.6	1.6	1.6	1.6	1.6	1.6	1.6	
Tyr	1	1.6	1.4	1.4	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	
Phe	1.1	1	1	1	1	1.1	1.1	1.1	1.1	1.1	1.1	1.1	

*R, remainder; P, endoform; M, major.

†Means not available because of technical problems of analysis.

TABLE 3. Relative amino acid of bitter-tasting fractions separated by ion exchange chromatography isolates expressed as groups and ratios.

Group or amino	Origins												
	R (C's' 71)			U (trial 2)			K 3			M-1			M-2
	Fractions												
	A	C	B	D	E	F	G	H	I	J	L	M	
Slices gel %													
Basic	5.6	2.1	0.0	2.0	1.0	1.0	1.0	2.6	1.0	1.0	1.0	1.2	
Acids	4.9	7.5	7.7	7.4	7.4	7.4	7.4	6.0	5.3	5.3	5.3	5.1	
All ph. a.c.	1.7	0.5	0.0	0.1	0.1	0.1	0.1	5.3	7.0	5.1	7.0	5.0	
Aromatic	4	1.5	1.7	1.1	1.1	1.1	1.1	1.1	1.6	1.6	1.6	1.1	
Hydroxy	4.1	4.1	7	3.8	3.8	3.8	3.8	3.4	5.4	5.4	5.4	4.0	
Acids	1.3	1.3	4	3	3	3	3	5	4	4	4	1.1	
Amino acids	1.4	1.1	1.2	2.0	2.0	2.0	2.0	2.0	1.3	1.3	1.3	1.2	

*R, other P's, trial 1, W. matter.

†Means for amino acids, average of trial 1, trial 2, trial 3, trial 4, trial 5.

lytic activity of extracted peptides and amino acids from bitter cheese with hydrolytic changes.

A characteristic elution pattern for each coagulant obtained by Sephadex gel filtration of the bitter concentrate extracted from our low pH cheeses indicated differences in their proteolytic abilities. The general order of elution (smallest to largest elution volume) of maximum bitterness was former, minor 1, minor 2, and endothis, thus indicating slightly larger components in former bitterness with decreasing size to those in endothis bitterness. Some overlapping of maximum bitterness areas, however, suggested relatively small differences of component sizes.

The wide elution range of strong bitterness with Gm. catalyst indicates a relatively large variation in size of bitter components produced. The bitter tasting fractions were bitter positive. Acid hydrolysis eliminated all bitterness, thus indicating it was from one or more peptides.

The bitter fractions from Sephadex gel filtration were resolved more fully by TLC. More than one area of bitterness was separated by TLC of the bitter Sephadex fraction for each coagulant. Apparently, then, more than one component in the fraction from Sephadex contributed to the bitter taste. The different relative migrations of the bitter tasting bands further indicated qualitative differences of the bitter components.

Contribution of free amino acids to bitter taste in our study probably was small because bitter tasting fractions were bitter positive, eluted comparatively rapidly from Sephadex G 25, and hydrolysis removed fraction bitterness.

Amino acids in bitter peptides isolated from cheese and enzymic hydrolysates of casein have been reported for the following cheeses: Gouda—proline, glutamic acid, alanine, valine, leucine, isoleucine, phenylalanine, aspartic acid, and small amounts of basic amino acids, methionine, threonine, serine, tyrosine, and tryptophan (15); Cheddar—glutamic acid, glycine, proline, valine, alanine, and leucine (20); spartan cheese, glutamic acid, glycine, alanine, valine, proline, tyrosine, and leucine or isoleucine (11); leucine, phenylalanine, methionine, tyrosine, proline, glutamic acid, alanine, threonine, glycine, serine, and arginine (6).

Most bitter fractions of this study were low in basic and aromatic amino acids (or high in acidic, aliphatic, and hydroxy amino acids). Factors in addition to these fractions were relatively large amounts of glutamic acid, proline, leucine, and valine and a fairly consistent ratio of aliphatic to acidic amino acids. In contrast, the bitter fractions from the wheats contained a relatively large amount of basic amino acids, especially lysine.

Wide combinations of amino acids in peptides apparently can cause a bitter taste, and no specific rules for characterizing bitter peptides are possible. Greater purification of bitter peptides and more amino acid sequencing will help resolve the contribution of structure to bitterness, particularly if compared to amino acid data from nonbitter peptides.

REFERENCES

1. Carr, J. W., T. C. Loughheed, and B. C. Baker. 1956. Studies on protein hydrolysis. IV. Further observations on the taste of enzymic protein hydrolysates. *J. Sci. Food Agric.* 7:629.
2. Crawford, R. J. M. 1977. Bitterness in cheese. Pages 1-20 in *Ann. Bul. Int. Dairy Fed., Brussels, Belgium*, No. 97.
3. Hanuman, J. S., R. D. H. L. H. Luyckx. 1974. A bitter peptide from Cheddar cheese. *Agric. Biol. Chem.* 38:375.
4. Harwalter, V. R. 1959. Comparative study of bitter fraction obtained from mozzarella and bitter Cheddar cheese. *J. Dairy Sci.* 53:956.
5. Harwalter, V. R. 1972. Influence of hydrogen ion concentration on extractability and flavor of free and astringent cheese components from Cheddar cheese and cultured curd. *J. Dairy Sci.* 55:742.
6. Harwalter, V. R., and J. A. Upton. 1971. Isolation of bitter and astringent fractions from Cheddar cheese. *J. Dairy Sci.* 54:8.
7. Hettings, D. H., and L. F. Farnelke. 1965. Amino acid sequences of fractions from bitter and non-bitter Kambs cheese. *J. Dairy Sci.* 48:764.
8. Ichikawa, K., I. Yamamoto, and T. Katsunoto. 1956. Studies on the bitter taste peptides produced by proteolyses. Part I. The isolation of bitter taste peptides by treatment of protease of *Staphylococcus aureus* and isolation of the peptide. *J. Agr. Chem. Soc. Japan* 33:1014.
9. Kikuchi, T., and S. Togo. 1970. Use of micro-Malvernity in cheese making. *Prod. Tech. Int. Dairy Congr.* 18:289.
10. Kosikowski, T. V. 1977. Cheese and fermented milk foods. Edwards Brothers, Inc., Ann Arbor, MI.
11. Kuster, H. W., Dornick, and K. Poggendorf. 1971. A new control method for the protein degradation in cheese. *Milchwissenschaft* 26:94.
12. Lawrence, R. C., L. K. Cranmer, J. Gies, and B. G. Mayers. 1972. Cheddar cheese flavor. I. The role of starter and rennet. *New Zealand J. Dairy Sci. Technol.* 7:33.
13. Lawrence, R. C., and R. C. Lawrence. 1972. Cheddar cheese flavor. IV. A new hypothesis to account for the development of bitterness. *New Zealand J. Dairy Sci. Technol.* 7:57.
14. Miles, G. D., and U. D. Thomas. 1980. Bitterness development in Cheddar cheese: effect of level of starter proteolysis. *New Zealand J. Dairy Sci. Technol.* 13:121.
15. Raovala, G. W. 1953. Bitter compounds from cheese. *Proc. XIII Int. Dairy Congr.* 2:676.
16. Richardson, H. C., and L. K. Cranmer. 1973. Casein proteolysis and bitter products in Cheddar cheese. *New Zealand J. Dairy Sci. Technol.* 8:46.
17. Schwaninger, H., and U. Behnke. 1975. Bitter peptides from casein and cheese III. Characterization of bitter peptides with identification of bitter taste. *Nahrung* 19:497.
18. Spackman, D. M., W. H. Stein, and S. Moore. 1958. Automatic recording apparatus for use in the chromatography of amino acids. *Anal. Chem.* 30:1190.
19. Sullivan, J. J., and G. R. Jags. 1970. A model for the formation and degradation of bitter peptides in cultured dairy products. *Australian J. Dairy Technol.* 25:111.
20. Windham, H. M. 1955. The influence of milk coagulating enzymes upon some of the constituents and flavor of Cheddar cheese during ripening. Ph.D. thesis, Cornell Univ., Ithaca, NY.