Bovine Milk κ-Casein Trypsin Digest Is a Growth Enhancer for the Genus *Bifidobacterium*

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Bifidobacterium bifidum and *Bifidobacterium longum*, both isolated from infant feces, were used as assay organisms to evaluate bifidobacterial growth-promoting activities of bovine milk casein components. κ-Casein was found to be the most potent growth enhancer when digested by trypsin. Its glycomacropeptide had no activity whatever. Activity was lost when the disulfide bonds in κ-casein were modified by performic acid oxidation, reduction–alkylation, dinitrophenylation, or combination in a mixed disulfide linkage with 2-mercaptopyridine. Synthetic κ-casein peptides containing cysteine or other cysteine compounds could not substitute for the κ-casein digest. Similar behavior was observed with yeast extract and hog gastric mucin.

INTRODUCTION

Bifidobacteria are anaerobic Gram-positive organisms that are important both physiologically and industrially for dairy products. Their properties have been amply reviewed in recent years (Bezkorovainy and Miller-Catchpole, 1989; Rasic and Kurmann, 1983). It is now well established that most species of the genus *Bifidobacterium* cannot grow in fully synthetic media and require such undefined biological products as bovine casein digest, bovine milk whey, hog gastric mucin, or yeast extract (Poch and Bezkorovainy, 1988; Petschow and Talbott, 1990). Active ingredients of these complex biological products have not been identified. This paper presents evidence that κ-casein is the microbial growth promoter in whole bovine milk casein and focuses on its disulfide/sulfhydryl residues as important biologically active components responsible for this phenomenon.

MATERIALS AND METHODS

Chemicals. All chemicals, enzymes, and proteins, including caseins and the glycomacropeptide of κ-casein, were purchased from Sigma Chemical Co. (St. Louis, MO), unless otherwise noted. Peptone and trypticase used to prepare the trypticase–peptone–yeast extract (TPY) medium (Scardovi, 1986) were purchased from BBL Microbiology Systems (Cockeysville, MD). Commercial κ-casein and glycomacropeptide were subjected to amino acid analysis, and the results of both corresponded to published figures (Ernstrom, 1974).

Peptides of κ-Casein. Anticipated tryptic peptides of κ-casein containing the two cysteine residues, i.e., Cys-Glu-Lys and Ser-Cys-Gln-Ala-Gln-Pro-Thr-Thr-Met-Ala-Arg, were synthesized by solid-phase methodology in the Protein Sequencing/Synthesis Laboratory of the University of Illinois at Chicago. Their amino acid compositions were confirmed by amino acid analysis.

Microorganisms and Their Growth. Two microorganisms originating from infant feces were used to assay for growth-promoting activities of various substances: *Bifidobacterium bifidum* (ATCC 15696) and *Bifidobacterium longum* (ATCC 15708). Cultures of these bacteria in the lyophilized form were purchased from American Type Culture Collection (Rockville, MD).

Growth of microorganisms in the presence of potential growth promoters was assayed in a fully synthetic medium as described previously (Poch and Bezkorovainy, 1988). This synthetic medium contained all amino acis including cystine, certain vitamins, and certain metals: iron, magnesium, and manganese. Growth was measured as turbidity (A) at 610 nm after 24 h. Growth was always compared to a reference standard: either growth in the TPY medium or growth in the synthetic medium, in the presence of specific amounts of trypsinized κ-casein. The formula

$$\text{growth (\%)} = \frac{A_{\text{test culture}} - A_{\text{synth medium}}}{A_{\text{ref std}} - A_{\text{synth medium}}} \times 100$$

was used to express growth, where $A_{\text{synth medium}}$ is turbidity obtained with the synthetic medium alone. A 5-mL culture of bifidobacteria with an $A_{610} = 1.0$ was equivalent to 2.75 mg dry weight (Bezkorovainy and Solberg, 1989). All assays were done several times in duplicate and data are presented (e.g., Tables I and II) with the appropriate standard deviations.

Analytical and Chemical Procedures. Amino acid analyses of proteins and peptides were performed in Professor Dubin's laboratory at the Hektoen Institute of Chicago following hydrolysis with 6 N HCl for 24 h at 100 °C. The Beckman Model 119CL analyzer was used. Carbohydrate analyses were performed by the phenol–H_2SO_4 method (Dubois et al., 1956). Protein analyses were done according to the method of Lowry et al. (1951). Sulfhydryl groups (following reduction with $NaBH_4$) were assayed by the Ellman reaction as described by Cavallini et al. (1966).

Tryptic digestion was performed in 0.01 M $NaHCO_3$ solution containing 0.001 M $CaCl_2$ at 37 °C. Protein concentration was 2.5 mg/mL, and trypsin concentration was $^1/_{20}$ that of the substrate. Progress of digestion was followed by the ninhydrin reaction according to the method of Troll and Cannan (1953). Cleavage of κ-casein by rennin was carried out by the method of Azuma et al. (1984).

Disulfide bonds were broken oxidatively with performic acid according to the method of Moore (1963). Disulfide bonds were also modified either by reduction only using 0.05–0.1 M mercaptoethanol, or 1 M $NaBH_4$, or by reduction–alkylation using mercaptoethanol and iodoacetic acid as described by Crestfield et al. (1963). Reduced sulfhydryl groups could be oxidized to disulfide residues by bubbling O_2 into a 0.1% (w/v) protein/peptide solution at pH 8.5 for 4 h at 4 °C with stirring. Following this treatment, no Ellman-positive groups could be detected. Alternatively, sulfhydryl groups were reoxidized to disulfide residues by tetranitromethane as described by Torchinsky (1981). Reduced κ-casein digests were reoxidized to make mixed disulfide residues as follows: the reduced digest in 0.1 M phosphate buffer at pH 7.5 was incubated in the presence of 0.1 M 2,2′-dithiodipyridine for 2 h at 25 °C with stirring. It was then dialyzed against water and lyophilized. To remove the 2-mercaptopyridine from the κ-casein digest and recover its SH residues, the latter was incubated with 0.1 M mercaptoethanol for 4 h at 37 °C under nitrogen. The mercaptoethanol and mercaptopyridine were then removed by dialysis. Methodology involving modification of SH residues by 2,2′-dithiodipyridine is described by Grasetti and Murray (1967) and by Carlsson et al. (1974).

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Table I. Bifidobacterial Growth-Promoting Activity of Tryptic Casein Digests and Components of κ-Casein[a]

casein digest	concn, mg/mL	growth-promoting act. *B. bifidum*	growth-promoting act. *B. longum*
trypsin	0.1	15.3 ± 2.7	9.9 ± 0.5
	0.25	15.4 ± 2.6	-
whole casein	2.0	33.4 ± 2.3	24.2 ± 4.5
	5.0	49.5 ± 2.9	26.8 ± 0.5
	10.0	-	40.9 ± 4.8
α-casein	2.0	19.1 ± 0.7	29.5 ± 0.9
	5.0	20.7 ± 1.2	24.1 ± 4.8
β-casein	2.0	39.0 ± 5.9	34.2 ± 4.1
	5.0	44.6 ± 2.2	31.2 ± 0.5
κ-casein	0.5	43.5 ± 4.3	32.4 ± 3.3
	1.0	68.9 ± 4.4	52.9 ± 3.5
	2.0	86.3 ± 8.6	77.9 ± 4.4
	5.0	93.0 ± 8.8	84.2 ± 4.0
p-κ-casein[b]	0.5	52.5 ± 10.0	52.5 ± 5.0
	1.0	77.2 ± 6.8	86.5 ± 11.9
glycomacropeptide (commercial)[b]	1.0	11.0 ± 1.3	9.0 ± 0.4
glycomacropeptide (prepared from κ-casein)[b]	1.0	30.4 ± 15	27.5 ± 6.3

[a] In percent of growth observed in the TPY medium (Scardovi, 1986). [b] Glycomacropeptides and p-κ-casein were treated with trypsin as the intact caseins. Untreated glycomacropeptide showed the same activity as its trypsinized counterpart.

Modification of tyrosyl residues with tetranitromethane was carried out as described by Mierzwa and Chan (1987). Tryptophan residues were modified by the bromine adduct of 2-(2′-nitrophenylsulfenyl)-3-methyl-3-bromoindolenine (BNPS-skatole) as described by Omenn et al. (1970). Tryptophan was then assayed as described by Chrastil (1986). Cyanogen bromide fragmentation of κ-casein was carried out as described by Bezkorovainy and Grohlich (1972). CNBr breaks those peptide bonds, where methionine residues contribute the carboxyl groups; methionine is modified in the process.

Desalting Procedures. Chemically or enzymatically modified protein and peptide samples had to be desalted to be assayed for microbial growth-promoting activity. Dialysis was performed wherever intact proteins were modified. Additionally, tryptic κ-casein digests could be dialyzed by using 1000-Da cutoff dialysis tubing without loss of activity. Small molecular weight substances were desalted on Sephadex G-10 (Pharmacia Corp., Piscataway, NJ) using water as an eluant.

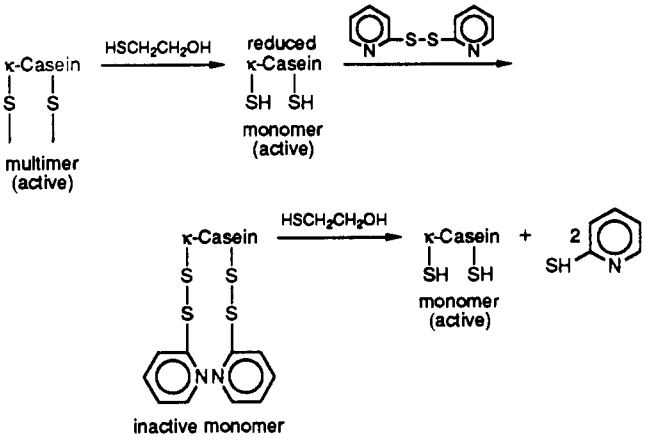
RESULTS

Enzymatic Digests of the Caseins. α-, β-, and κ-caseins were digested by trypsin using a 1:20 enzyme:substrate ratio. Digestions were followed by analysis of ninhydrin-positive material until no further increase of chromogen was observed. This took a minimum of 2 h at 37 °C. Addition of fresh samples of the enzyme to such digests failed to increase chromogen concentration, indicating that digestion was complete. Intact caseins showed no bacterial growth-promoting activity (data not shown). Trypsin digests, however, were minimally active in the case of α- and β-caseins and fully active with κ-casein (Table I). Growth of organisms in the presence of 5 mg/mL κ-casein approached that observed in the TPY medium. Moreover, κ-casein digests behaved in a dose-dependent manner, as indicated in Table I.

Bovine κ-casein was subjected to rennin (chymosin) digestion, and both the p-κ-casein (insoluble) and glycomacropeptide fractions were isolated. As seen in Table I, most of the activity resided in the p-κ-casein fraction, whereas little was present in the carbohydrate-containing glycomacropeptide. Even less growth-promoting activity was observed in commercially prepared (from whole bovine milk casein) glycomacropeptide or the same material prepared by the authors from whole bovine milk casein. The activity in the glycomacropeptide prepared from κ-casein may be explained by the presence of p-κ-casein impurities therein: test for sulfhydryl groups via the Ellman reagent indicated the presence of 0.036 μmol of SH/mg (0.075 μmol/mg in reduced κ-casein). The glycomacropeptide portion of κ-casein should have no cysteine residues, and indeed none was found in the commercial glycomacropeptide or in glycomacropeptide prepared by the authors from whole bovine milk casein.

Role of Sulfhydryl Groups in Growth-Promoting Activity of κ-Casein. It was next of interest to determine which component(s) of p-κ-casein was (were) essential for its microbial growth-promoting activity. κ-casein was subjected to various procedures that modify the disulfide bonds. It should be noted that κ-casein contains two cysteine residues—in positions 11 and 88 of the molecule. κ-Casein itself is believed to consist of disulfide-linked multimers of the basic 169 amino acid unit (Ernstrom, 1974). Table II shows the results of disrupting this disulfide bond arrangement.

First, it is seen that a simple reduction of the S–S bonds was not accompanied by a loss of activity of the κ-casein digest. However, if the SH groups were oxidized with performic acid or H_2O_2 alone, activity declined. The same was observed when the reduced κ-casein was alkylated with iodoacetic acid or dinitrophenylated with fluorodinitrobenzene at pH 5. Alkylation only with iodoacetate under the same conditions did little, nor did simple exposure to concentrated formic acid. Activity was lost when reduced κ-casein was treated with 2,2′-dithiodipyridine, though activity was restored when the SH blocking agent was removed by mercaptoethanol. The pertinent set of reactions can be envisioned as follows:



Mercaptoethanol per se, at concentrations used, had no growth-enhancing or growth-inhibiting effects on bifidobacteria.

Since performic acid oxidation is known to affect other groups as well, especially tryptophan, tyrosine, and methionine, chemical modification procedures were performed on these groups to assess their possible role as growth promoters. Results are presented in Table II, from which it is seen that only small amounts of activity were lost when tyrosine, methionine, or tryptophan residues were modified. These results strengthen the hypothesis that disulfide/sulfhydryl residues are involved in bifidobacterial growth-promoting activity.

Growth-Promoting Activities of Other Cysteine-Containing Substances. Results in Table II have

Table II. Bidifobacterial Growth-Promoting Activities of κ-Casein Digests following Modification of Various Amino Acid Side Chains[a]

modification	group modified	% groups present compared to unmodified κ-casein	growth-promoting act.	
			B. bifidum	*B. longum*
performic acid oxidation	cysteine/cystine[b]	37.7 ± 2.7	5.4 ± 2.5	30.5 ± 9.6
performic acid oxidation[c]	cysteine/cystine	22.3 ± 0.4	3.8 ± 0.2	9.3 ± 2.2
reduction-alkylation	cysteine/cystine	40.5 ± 1.0	31.7 ± 6.1	39.9 ± 6.4
reduction-alkylation[c]	cysteine/cystine	29.3 ± 2.0	20.8 ± 8.5	29.6 ± 3.8
formic acid only	none	103 ± 3.6[b]	116.9 ± 14.1	119.7 ± 14.1
H_2O_2 only	partial cystine/cysteine	57.2 ± 1.0	78.2 ± 5.7	69.3 ± 12.9
mercaptoethanol only (0.1 M)	cystine	100.0 ± 0.0	100.2 ± 13.6	69.3 ± 6.1
$NaBH_4$ (1 M) (reduced)	cystine	100.0 ± 0.0	114.0 ± 4.9	111.0 ± 11.0
iodoacetate only (0.2 M)	various groups	98.8 ± 5.8	88.1 ± 9.4	99.1 ± 6.2
reduced, FDNB treated[d]	cysteine/cystine	39.0 ± 1.4	22.2 ± 1.0	29.0 ± 7.7
2,2'-dithiodipyridine	cysteine/cystine	190 ± 40[e]	32.1 ± 6.8	25.8 ± 1.4
2,2'-dithiodipyridine, then deblocked	cysteine/cystine	85.9 ± 4.1	88.3 ± 11.0	109.0 ± 4.2
BNPS-skatole	tryptophan	85.1 ± 8.4,[b] 20.6 ± 0.2[f]	69.3 ± 6.1	73.9 ± 9.3
tetranitromethane	tyrosine	109.5 ± 8.0[b]	73.5 ± 6.4	121.5 ± 5.5
CNBr[g]	methionine	-	105.4 ± 4.2	117.6 ± 5.1
precipitate only	methionine	-	127.3 ± 20.4	127.7 ± 39.1
soluble fraction only	methionine	-	53.3 ± 2.8	52.2 ± 5.1

[a] Unless otherwise noted, tryptic digestion was carried out after chemical modification. In percent growth observed with an equivalent quantity of unmodified κ-casein tryptic digest. [b] Disulfide bonds were assayed as SH groups by the Ellman reagent following reduction with $NaBH_4$. [c] Digested with trypsin first and then modified. [d] Reaction at pH 5 following reduction of SS residues by $NaBH_4$. Fluorodinitrobenzene (FDNB) does not react with NH_2 or imidazole residues at this pH (Torchinsky, 1981). [e] This mixed disulfide-containing κ-casein should have twice as many releasable sulfhydryl groups as native κ-casein. After the 2-mercaptopyridine is deblocked and removed, that quantity should return to normal. [f] Tryptophan residues. [g] CNBr cleaves the κ-casine molecule into an insoluble fraction, very similar to *p*-κ-casein, and a soluble fraction roughly equivalent to the glycomacropeptide. Methionine is lost in the process.

Table III. Substances and Combinations Thereof That Showed Less than 10% of the Bifidobacterial Growth-Promoting Activity Observed with 2 mg of κ-Casein Trypsin Digest[a]

	substance(s)[b]		substance(s)[b]
1	myoglobin	13	chloroform–methanol (2:1) extract[c]
2	lactoglobulin		
3	insulin	14	Cys-Glu-Lys (reduced and oxidized)
4	bovine serum albumin	15	Ser-Cys-Gln-Ala-Gln-Pro-Thr-Thr-Met-Ala-Arg (reduced and oxidized)
5	IgG		
6	Cysteine, cystine		
7	Phe-Cys-Cys-Phe	16	combinations of 14 and 15, reduced and oxidized
8	Gly-Cys-Cys-Gly		
9	lipoic acid	17	combinations of 14, 15, Ca, Zn, Cu, and Cr
10	ascorbic acid		
11	glutathione, oxidized and reduced	18	combinations of 14, 15, 9, 10, and 13
12	Ca^{2+}	19	combinations of 14, 15, and the glycomacropeptide

[a] Quantities of substances used were equivalent to 2 mg/mL of trypsinized κ-casein. [b] All proteins (items 1–5) were tryptic digests. [c] Extract from 20 mg of whole bovine milk casein.

Table IV. Chemical Manipulation of κ-Casein That Did Not Substantially Alter Its Microbial Growth-Promoting Activity following Tryptic Digestion[a]

procedure	act.	
	B. bifidum	*B. longum*
exhaustive dialysis	105.8 ± 4.4	109.9 ± 9.1
exhaustive dialysis under reducing conditions (0.05 M mercaptoethanol)	76.5 ± 2.1	91.8 ± 0.4
EDTA-treated[b]	163.3 ± 1.8	134.5 ± 6.3
extracted with butanol[c]	100.4 ± 3.7	106.6 ± 6.1
extracted with chloroform–methanol (2:1)[c]	84.8 ± 5.1	91.0 ± 8.9

[a] Activity is in percent of that obtained with an equivalent amount of unaltered κ-casein tryptic digest. [b] Ethylenediamine tetraacetic acid; conditions were the following: 0.5% (w/v) EDTA, 1.0% (w/v) κ-casein digest, 4 M urea, pH 7.0, 4 °C, 16 h. The EDTA and urea were removed by dialysis. [c] These experiments were done on whole milk casein.

suggested that κ-casein may act as a simple provider of cysteine residues for the growth of bifidobacteria. If that were the case, then additional cysteine or cystine, or peptides and proteins carrying cysteine, should be able to substitute for κ-casein as bifidobacterial growth promoters. Various cysteine-containing substances, listed in Table III, were tested with the synthetic medium for bifidobacterial growth-promoting activity. Quantities used were adjusted to simulate cystine/cysteine content in 2 mg/mL of κ-casein. No activity was found in any of these substances.

Of special interest were synthetic cysteine-containing peptides prepared on the basis of amino acid sequences of peptides potentially obtainable from tryptic digestion of κ-casein: Cys-Glu-Lys, corresponding to amino acids 11–13 of κ-casein, and Ser-Cys-Gln-Ala-Gln-Pro-Thr-Thr-Met-Ala-Arg, corresponding to amino acids 87–97 of κ-casein. These synthetic peptides were used singly, in combination, and in reduced and oxidized forms, the latter being accomplished either by O_2 or tetranitromethane. Neither these synthetic peptides nor other sulfur amino acid carrying substances could substitute for the tryptic κ-casein digests.

To explore the further possibility that such substances merely supply cysteine to bifidobacteria, the cystine was eliminated from the synthetic medium used for assays and substituted by the κ-casein digest (1–5 mg/mL) in the synthetic medium. Growth-promoting activities of such media were 7.1 and 11.7% for *B. bifidum* and *B. longum*, respectively, on the basis of growth obtained with 1 mg/mL trypsinized κ-casein in the complete synthetic medium. Moreover, in combination with tryptic digests of α- and β-caseins, these synthetic peptides showed no increase in activity beyond that ascribable to α- and β-caseins alone (Table I).

A determination was made of whether or not amino acid-carbohydrate mixtures simulating κ-casein composition would substitute for trypsinized κ-casein as growth promoters. A synthetic mixture was made of all such amino acids plus galactose, galactosamine, and sialic acid (filter sterilized), and an equivalent of 2.0 mg/mL κ-casein was then used in growth assays. Additionally, κ-casein was hydrolyzed with 6 N HCl for 24 h at 100 °C and the hydrolysate tested for growth-promoting activity. Activities with

Table V. Fractionation of κ-Casein Tryptic Digests by Various Procedures

procedures and fractions	protein recovery,[a] % applied	carbohydrate content, %	sulfhydryl residues[b]	act.[b] *B. bifidum*	act.[b] *B. longum*
dialysis (3.5-kDa cutoff)					
diffusible fraction	40.5	2.2	66.0 ± 2.2	44.9 ± 10.0	60.0 ± 0
nondiffusible fraction	51.4	4.6	133.0 ± 3.4	142.5 ± 7.3	142.9 ± 1.0
gel filtration (Sephadex G-25) of nondiffusible fraction[c]					
50–65 mL	51.6	–	32.3 ± 1.5	65.1 ± 4.0	107.0 ± 2.0
66–100 mL	24.2	–	31.2 ± 1.4	97.9 ± 2.5	110.3 ± 4.4
100–145 mL	24.2	–	133.4 ± 6.8	146.7 ± 16.1	169.7 ± 1.1
gel filtration (Bio-Gel P-4) of κ-casein tryptic digest[d]					
50–103 mL	57.4	–	101.2 ± 3.2	105.9 ± 6.0	125.1 ± 4.9
104–128 mL	12.4	–	34.5 ± 3.7	94.1 ± 2.6	81.5 ± 2.8
129–170 mL	28.8	–	22.6 ± 0.0	60.2 ± 3.7	54.6 ± 1.6
171–200 mL	1.5	–	0.0	27.0 ± 5.1	32.5 ± 2.1

[a] On the basis of the Lowry reaction. [b] In percent compared to an identical weight of κ-casein tryptic digest. [c] Column 2 × 35 cm, void volume 55 mL, salt volume 260 mL, flow rate 0.5 mL/min, eluant 0.05 M ammonium acetate at pH 8.5. [d] Column 2 × 35 cm, void volume 57 mL, salt volume 227 mL, flow rate 0.4 mL/min, eluant as in footnote *c*.

Table VI. Growth-Promoting Activities of Performic Acid Oxidized Bifidobacterial Growth-Enhancing Substances in Relation to Their Unmodified Counterparts

substance	concn, mg/mL	releasable sulfhydryl groups (in % found in the unmodified substance)[a]	act. *B. bifidum*	act. *B. longum*
whole bovine milk casein tryptic digest	10	13.8[b]	23.2 ± 4.6	38.6 ± 2.9
hog gastric mucin	10	29.5	42.1 ± 13.3	23.0 ± 0.2
yeast extract	5	21.6	7.1 ± 0.8	10.6 ± 4.6

[a] Done by the Ellman reaction after reduction. [b] Whole bovine milk casein digest had about 27% sulfhydryl groups of κ-casein on a w/w basis.

B. longum were 9.6 ± 2.3 and 3.4 ± 6.6% for the hydrolysate and the synthetic mixture, respectively, compared to 2.0 mg/mL trypsinized κ-casein. Similar results were obtained with *B. bifidum*.

Possible Role of κ-Casein as a Nutrient Carrier. The possibility exists that either κ-casein is a binder of a substance or substances not present in the synthetic medium and required by bifidobacteria for growth or, alternately, κ-casein cysteine-containing peptides may be required as membrane carriers of nutrients either present in or absent from the synthetic medium. An X-ray fluorescence metal analysis (Sky-Peck and Joseph, 1981) of κ-casein was kindly performed by Dr. Howard Sky-Peck, and the following metals were found in Sigma's κ-casein preparations, with quantities indicated on a mole/mole basis: Ca, 0.4; Fe, 0.084; Cu, 0.0046; Cr, 0.0029; and Zn 0.015. Trace amounts (less than 0.001 mol/mol of κ-casein) of Ni, Se, Mn, Rb, Sr, Pb, and Hg were also present. Fe and Mn were components of the synthetic medium (Poch and Bezkorovainy, 1988), and should the cysteine peptides have been required as their carriers, the synthetic tri- and undecapeptides would have shown growth-promoting activity. Zn, Ca, Cu, and Cr were not specifically used in making up the synthetic medium. But when metals were stripped from κ-casein by EDTA, no loss of activity was observed and, in fact, mild growth stimulation was present (Table IV).

To remove other substances that may be bound to κ-casein and which could account for its activity, exhaustive dialysis and extraction of whole milk casein with organic solvents was performed. There was no substantial decrease in activity (Table IV). There was no activity observed with thioctic (lipoic) or ascorbic acid singly or in combination with cysteine-containing κ-casein peptides (Table III).

Attempts To Isolate Biologically Active Peptides from Trypsinized κ-Casein. Numerous attempts were made to fractionate κ-casein digests for the purpose of isolating biologically active components. Such separations must be done on a macro scale, since relatively large quantities (1–5 mg/mL culture) of peptide material is required for growth assays. It was first discovered that a preliminary separation could be made by exhaustive dialysis of κ-casein tryptic digests against H_2O using dialysis tubing with a stated molecular weight cutoff of 3500 (Spectrum Medical Inc., Los Angeles, CA). Following such dialysis, the nondiffusible fraction was chromatographed on Sephadex G-25 columns (Pharmacia Corp., Piscataway, NJ). The results are depicted in Table V, which indicates that though dialysis did concentrate the growth-promoting activity somewhat, further purification by gel filtration did not result in a substantive increase in activity of any fraction. Similar results were obtained when κ-casein tryptic digests were chromatographed directly on Bio-Gel P-4 columns. Nevertheless, increased biological activity was always associated with high sulfhydryl group content.

Disulfide Bonds of Whole Milk Casein, Hog Gastric Mucin, and Yeast Extract. It was previously reported that, in addition to bovine casein tryptic digests, hog gastric mucin and yeast extract also possessed bifidobacterial growth-promoting activities (Poch and Bezkorovainy, 1988). These mixtures were also oxidized by performic acid, and, as Table VI indicates, there were considerable losses of activity in all samples tested.

DISCUSSION

Since the time when Kehagias et al. (1977) showed that partial acid hydrolysate of bovine milk casein was stimulatory to the growth of *Bifidobacterium bifidum* var. pennsylvanicus, it was demonstrated that other bifidobacterial species, be they from human or animal sources, also require biologically complex materials for growth. Our laboratory has focused on bovine casein as a source of such factor or factors. This paper reports that the growth-promoting activity resides in the *p*-κ-casein portion of the κ-casein molecule; i.e., the carbohydrate portion of the molecule has nothing to do with this activity. This is

contrary to the findings when the *human* κ-casein glycomacropeptide (Azuma et al., 1984) was used, though note should be taken to the fact that bovine and human κ-casein glycomacropeptides are chemically very different. Furthermore, it was shown that either the disulfide or sulfhydryl residues of κ-casein were essential for its activity. Given these facts, it would not be difficult to imagine why α- and β-casein digests are relatively inactive: they do not contain cystine. Moreover, one might image that the κ-casein simply provides cysteine/cystine for the growth of the microorganism, where free cysteine may perhaps not be easily transported into the organism. But that did not prove to be the case: glutathione or other cysteine/cystine peptides could not repalce the κ-casein digest, nor could the κ-casein digest replace the cystine present in the synthetic medium. Disappointingly, the two cysteine-containing tryptic peptides, synthesized on the basis of the κ-casein amino acid sequence, were not able to replace the κ-casein digest either, nor were they active in the presence of α- and β-casein tryptic digests. It thus does not appear that tryptic peptides of κ-casein were simply amino acid suppliers for the growth of bifidobacteria, as was the case with the lactobacilli (Kihara and Snell, 1960).

The type of growth-promoting behavior observed in this work may be explained first of all by the possibility that κ-casein is a carrier for an as yet unidentified bifidobacterial growth factor. This factor may, in fact, be associated with the disulfide residues and may be displaced therefrom by oxidative and/or alkylation procedures. We have not been able to identify any such factor from among the more common metals, vitamins, or lipids. Another possibility is that another tryptic peptide, along with the cysteine-containing peptide or peptides, is required for growth. This is perhaps the more viable alternative, since peptide separation was always accompanied by an overall loss of activity. To test this hypothesis, however, it will be necessary to synthesize all tryptic peptides of *p*-κ-casein and test for their growth-promoting activity singly and in combination with the cysteine-containing peptides.

Lastly, it is possible to conclude that the basis for microbial growth-promoting activity in casein digests, hog gastric mucin, and yeast extract may be the same: the disulfide/sulfhydryl residues in combination with something else. We make this conclusion on the basis of the fact that activity was lost by these factors when they were modified by performic acid oxidation of their constituent sulfur amino acids.

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LITERATURE CITED

Azuma, N.; Yamauchi, K.; Mitsuoka, T. Bifidus growth-promoting activity of a glycomacropeptide derived from human κ-casein. *Agric. Biol. Chem.* **1984**, *48*, 2159–2162.

Bezkorovainy, A.; Grohlich, D. Cleavage of transferrin by cyanogen bromide. *Biochim. Biophys. Acta* **1972**, *263*, 645–650.

Bezkorovainy, A.; Miller-Catchpole, R. *Biochemistry and Physiology of Bifidobacteria*; CRC Press: Boca Raton, FL, 1989.

Bezkorovainy, A.; Solberg, L. Ferrous iron uptake by *Bifidobacterium breve*. *Biol. Trace Elem. Res.* **1989**, *20*, 251–267.

Carlsson, J.; Axén, R.; Brocklehurst, K.; Crook, E. M. Immobilization of urease by thiol-disulphide interchange with concomitant purification. *Eur. J. Biochem.* **1974**, *44*, 189–194.

Cavallini, D.; Graziani, M. T.; Dupré, S. Determination of disulfide groups in proteins. *Nature* **1966**, *212*, 294–295.

Chrastil, J. Spectrophotometric determination of tryptophan and tyrosine in peptides and proteins based on new color reactions. *Anal. Biochem.* **1986**, *158*, 443–446.

Crestfield, A. M.; Moore, S.; Stein, W. H. The preparation and enzymatic hydrolysis of reduced and S-carboxymethylated proteins. *J. Biol. Chem.* **1963**, *238*, 622–627.

Dubois, M.; Gilles, K. A.; Hamilton, J. K.; Rebers, P. A.; Smith, F. Colorimetric method for determination of sugars and related substances. *Anal. Chem.* **1956**, *28*, 350–356.

Ernstrom, C. A. Milk-clotting enzymes and their action. In *Fundamentals of Dairy Chemistry*; Webb, B. H., Johnson, A. H., Alford, J. A., Eds.; Avi Publishing: Westport, CT, 1974; p 700.

Grassetti, D. B.; Murray, J. F., Jr. Determination of sulfhydryl groups with 2,2'- or 4,4'-dithiodipyridine. *Arch. Biochem. Biophys.* **1967**, *119*, 41–49.

Kehagias, C.; Jao, Y. C.; Mikolajcik, E. M.; Hansen, P. M. T. Growth response of *Bifidobacterium bifidum* to a hydrolytic product isolated from bovine casein. *J. Food Sci.* **1977**, *42*, 146–152.

Kihara, H.; Snell, E. E. Peptides and bacterial growth. VIII. The nature of streptogenin. *J. Biol. Chem.* **1960**, *235*, 1409–1414.

Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265–275.

Mierzwa, S.; Chan, S. K. Chemical modification of human α-proteinase inhibitor by tetranitromethane. *Biochem. J.* **1987**, *246*, 37–42.

Moore, S. On the determination of cystine as cysteic acid. *J. Biochem. Chem.* **1963**, *238*, 235–237.

Omenn, G. S.; Fontana, A.; Anfinsen, C. B. Modification of the single tryptophan residue of staphylococcal nuclease by a new mild oxidizing agent. *J. Biol. Chem.* **1970**, *245*, 1895–1902.

Petschow, B. W.; Talbott, R. D. Growth promotion of *Bifidobacterium* species by whey and casein fractions from human and bovine milk. *J. Clin. Microbiol.* **1990**, *28*, 287–292.

Poch, M.; Bezkorovainy, A. Growth-enhancing supplements for various species of the genus *Bifidobacterium*. *J. Dairy Sci.* **1988**, *71*, 3214–3221.

Rasic, J. L.; Kurmann, J. A. *Bifidobacteria and their role. Microbiological, nutritional-physiological, medical and technological aspects and bibliography*; Experientia Supplement 39; Birkhauser Verlag: Basel, 1983.

Scardovi, V. Genus Bifidobacterium Orla Jensen 1924, 472. In *Bergey's Manual of Systematic Bacteriology*; Sneath, P., Ed.; Williams & Wilkins: Baltimore, MD, 1986, Vol. 2.

Sky-Peck, H. H.; Joseph, B. Determination of trace elements in human serum by energy dispersive x-ray fluorescence. *Clin. Biochem.* **1981**, *14*, 126–131.

Torchinsky, Yu. M. *Sulfur in Proteins*; Pergamon Press: New York, 1981.

Troll, W.; Cannan, R. K. Modified photometric ninhydrin method for analysis of amino and imino acids. *J. Biol. Chem.* **1953**, *200*, 803–811.

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