Amino acid composition of casein isolated from the milks of different species

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Casein was isolated from the milks of the following species: cow, horse, pig, reindeer, caribou, moose, harp seal, musk-ox, polar bear, dall sheep, and fin whale. The caseins were subjected to acid hydrolysis, the resultant amino acids were converted to their *n*-butyl-*N*-trifluoroacetyl esters, and the amino acid composition of the caseins was determined by gas chromatographic analysis of these esters. Notable among the results was the close similarity, with respect to amino acid composition, of reindeer and caribou caseins. The results of the amino acid analyses of the other caseins are presented and discussed.

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On a prélevé la caséine du lait de plusieurs espèces: la vache, le cheval, le porc, le renne, le caribou, l'orignal, le phoque du Groënland, le bœuf musqué, l'ours polarie, le mouflon de Dall et le rorqual commun. On a soumis les différentes caséines à une hydrolyse à l'acide; les acides aminés résultant de l'hydrolyse ont été ensuite convertis en leurs esters *n*-butyl-*N*-trifluoroacétyliques. La composition de chaque caséine en acides aminés a été déterminée par l'analyse des esters par chromatographie en phase gazeuse. Parmi les résultats, on note la similitude frappante entre les caséines du renne et du caribou, en ce qui a trait à leur composition en acides aminés. On présente les résultats obtenus à l'analyse des acides aminés des autres caséines et on les discute.

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Introduction

The amino acid composition of bovine casein has been the subject of several previous investigations (3, 4, 10, 13, 16, 19, 22, 30, 45). Several workers have analyzed the caseins from milks of other domestic animals such as the buffalo (10, 22) and the goat (10, 19, 22, 27, 30). Various analytical techniques for the determination of amino acids were employed in these studies, including chemical and microbiological methods (13), paper chromatography (4, 10, 22), thin layer chromatography (16), and ion-exchange chromatography (27, 33, 35, 39). Data pertaining to the amino acid composition of equine and porcine caseins, unlike bovine casein, are scant or possibly non existent.

Data pertaining to the amino acid composition of casein from milks of species other than domestic species, including Arctic and sub-Arctic mammals, are very scarce. Ashworth *et al.* (3) analyzed by ion-exchange chromatography (35), the casein isolated from the milk of the Northern fur seal (*Callorhinus ursinus*) and found it to be similar in composition to bovine casein, except for its content of the dicarboxylic acids. Luhtala *et al.* (29) analyzed by ion-exchange chromatography (33), the "protein" of reindeer milk. The authors did not indicate clearly whether the term "protein" referred to casein or to casein plus whey proteins.

Table 1 shows the amino acid composition of bovine casein as determined by some earlier workers and Table 2 shows the amino acid composition of casein isolated from milks of species other than *Bos taurus*.

Several workers (2, 7, 8, 11, 20, 23, 24, 31, 36, 37, 40, 46) have suggested procedures for the preparation of amino acid derivatives and their separation by gas chromatography. The procedure employed in the present work for amino acid analysis comprised the derivatization method of Roach and Gehrke (36) and a modification of the method of Hagen and Black (15), in which calibration curves were prepared for each amino acid. The latter workers subjected samples that contained different weights of each amino acid

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TABLE 1. Amino acid composition (g/100 g) of bovine whole casein as reported by different workers

	Reference No.									
Amino acid	30	13	13[a]	10	22	4	41	16[a]	16	45
Alanine	2.3	3.0	3.5	2.98	2.3	3.84	3.31	3.0	3.3	2.3
Arginine	3.76	4.1	4.0	4.71	1.9	4.48	3.72	3.8	3.9	3.9
Aspartic acid	5.8	7.1	7.2	28.6[b]	3.5	7.63	6.95	7.6	7.3	4.2
Cystine	0.34	0.34	0.34	—	—	—	—	0.4	1.4	0.4
Glutamic acid	21.7	22.4	22.0	28.6[b]	11.8	20.8	20.8	22.0	20.8	21.9
Glycine	0.4	2.7	1.9	8.90[c]	1.6	2.05	1.97	2.4	2.2	0.4
Histidine	2.25	3.1	3.2	1.38	2.9	3.67	3.00	3.1	3.2	2.0
Isoleucine	6.0	6.1	7.6	13.5[d]	—	5.77	5.91	5.6	5.0	5.2
Leucine	10.8	9.2	10.3	13.55[d]	—	10.6	9.39	8.8	8.2	14.4
Lysine	6.85	8.2	8.3	8.47	5.8	7.35	7.68	7.8	8.1	6.0
Methionine	2.88	2.8	3.1	2.48	—	3.32	2.94	2.9	3.1	3.1
Phenylalanine	5.54	5.0	5.5	4.99	—	5.19	4.82	4.8	5.0	5.5
Proline	9.8	11.3	11.6	—	—	9.84	10.4	10.1	10.5	8.1
Serine	5.45	6.3	5.9	8.90[c]	4.0	4.64	5.67	6.4	6.3	5.0
Threonine	4.35	4.9	4.6	4.31	2.7	4.43	4.26	5.5	6.9	4.6
Tyrosine	5.99	6.3	6.2	4.80	3.1	6.04	5.62	5.8	4.9	5.5
Tryptophan	1.22	1.2	1.2	1.30	—	1.35	1.24	—	—	1.3
Valine	6.64	7.2	7.2	6.58	—	6.84	6.79	6.5	6.3	5.3

[a]Values cited from the literature by the author.
[b]Glutamic acid + aspartic acid.
[c]Glycine + serine.
[d]Isoleucine + leucine.

TABLE 2. Amino acid composition (g/100 g) of casein isolated from the milks of different species as reported by other workers

	Reference Nos. for:										
	Goat			Buffalo		Human			Northern fur seal	Rat	Reindeer
Amino acid	22[a]	27[b]	30	10[a]	22[a]	34[b]	1[b]	30, 45	3[b]	12	29[b,g]
Alanine	1.9	2.65	1.8	2.37	2.0	3.00	2.8	2.0	3.9	—	2.92
Arginine	1.9	1.34	2.66	2.78	1.2	2.79	2.05	3.43	4.2	3.42	3.58
Aspartic acid	5.2	7.62	1.7	27.3[d]	3.6	6.82	7.5	4.6	6.6	5.78	5.99
Cystine	—	T[c]	T[c]	—	—	0.48	0.95	0.64	1.78	3.99	0.74
Glutamic acid	11.3	18.2	12.1	27.3[d]	9.3	21.9	18.3	20.9	16.9	20.2	21.7
Glycine	1.2	1.41	—	8.85[e]	1.3	1.37	1.8	0.0	1.3	1.65	2.12
Histidine	3.5	3.65	1.85	1.62	2.9	2.47	2.15	1.77	2.4	2.50	3.39
Isoleucine	—	5.24	—	13.3[f]	—	5.90	4.4	6.3	4.2	4.67	4.34
Leucine	—	9.43	11.4	13.3[f]	—	10.4	10.8	12.2	8.9	5.54	8.95
Lysine	6.8	5.24	3.7	7.56	5.5	5.79	3.85	5.4	5.0	5.28	10.3
Methionine	—	1.35	—	2.01	—	2.05	0.7	2.4	2.08	1.87	2.27
Phenylalanine	—	3.77	2.0	4.46	—	3.56	3.8	5.8	3.7	3.75	4.38
Proline	—	8.29	6.9	—	—	13.9	10.9	8.9	8.1	7.84	8.79
Serine	4.4	4.10	0.7	8.85[e]	3.9	4.25	4.3	5.4	2.4	—	5.18
Threonine	2.4	4.38	—	3.74	2.1	4.05	4.35	4.5	3.5	4.49	4.24
Tyrosine	5.0	3.22	4.87	4.21	—	5.28	5.1	5.7	3.9	3.85	5.28
Tryptophan	1.5	—	1.37	1.45	—	1.06	2.4	1.4	—	4.57	—
Valine	—	6.47	6.0	5.58	—	7.48	4.85	5.0	5.9	5.02	5.89

[a]Values obtained by paper chromatography.
[b]Values obtained by amino acid analyzer.
[c]T = trace.
[d]Aspartic acid + glutamic acid.
[e]Glycine + serine.
[f]Isoleucine + leucine.
[g]Authors reported these values as amino composition of reindeer milk protein.

to an esterification and acylation procedure, which gave the *N*-trifluoroacetyl methyl amino acid esters. The individual mixtures of esters were dissolved in methanol and the volume of the resultant solutions was adjusted to a fixed value. Equal volumes of each solution were injected into the gas chromatograph. The authors found a linear relationship between the amount of amino acid injected and the corresponding peak area (as expressed in disc-integrator units). They postulated that the reason for this linear relationship was either that the conversion of the amino acid to its volatile derivative was complete, or that a constant proportion of each amino acid was converted to its volatile derivative, regardless of the amount of amino acid that was esterified. In the present study, the *ratios* of the peak areas (AAA) given by a suitable range of amounts of each amino acid, to the peak area (AIS) given by a set amount of internal standard (*n*-butyl stearate), were plotted against the amounts of that amino acid. Thus, unavoidable losses owing to incomplete transfers of the reaction mixtures during the course of esterification and acylation could be ignored. This procedure also eliminated the necessity of developing factors with regard to the yields of individual derivatives. Before the application of this method to the amino acid analysis of the casein sample, the method was applied to the analysis of several standard proteins. The values that were obtained (25) compared favourably with the results obtained previously by Gehrke *et al.*, who used gas chromatographic analyses and with the results of other workers (9, 14, 17, 18, 28, 41, 42, 43), who used conventional methods.

Materials

Milk samples (50–100 ml) were obtained from the following domestic, Arctic, and sub-Arctic species: cow (*Bos taurus*), horse (*Equus caballus*), pig (*Sus scrofa*), reindeer (*Rangifer tarandus*), caribou (*Rangifer tarandus*), moose (*Alces alces*), musk-ox (*Ovibos moschatus*), harp seal (*Pagophilus groenlandicus*), polar bear (*Thalarctos maritimus*), dall sheep (*Ovis dalli dalli*), and fin whale (*Balaenoptera physalus*). Casein was precipitated from these milks by acid precipitation at pH 4.6 (44). The details of the procedure, along with the particulars of nitrogen determination, are given in a previous publication (5).

Methods

Gas Chromatograph

A dual-column F and M model 810 gas chromatograph equipped with dual hydrogen flame ionization detectors and a Minneapolis–Honeywell (Brown) recorder (−0.2 to 1.0 mV) was used in the amino acid analyses. Compressed gases (nitrogen, air, and hydrogen) were obtained from Ohio Medical Products Canada Ltd. Drying tubes containing 'tell tale' silica gel G and Linde molecular sieve 5A (Hewlett–Packard, Avondale Division, Avondale, Pa.; catalogue No. 8501–5208) were inserted in each of the gas lines. Single column operation was employed for the amino acid analyses. The glass column (8 ft × ¼ in. (1 ft = 30.48 cm)) was packed with neopentylglycol sebacate (NPGSeb; 0.5%) on acid-washed Chromosorb G (80/100 mesh). The tube was not packed in the region that was inserted into the injection port. The brass Swagelok fittings were coated with Teflon ('Fluoroglide,' Chemplast, Inc., Wayne, N.J., U.S.A. 97470). The column was conditioned overnight (12–15 h) at 210 °C.

The chromatographic conditions that were employed are as follows: column temperature, 67 °C (initial), 210 °C (final); program rate, 4 °C/min; detector temperature, 250 °C; injection port temperature, 230 °C; range, 10; attenuation, 2; chart speed, 0.25 in./min; carrier gas (N_2) flow rate, 60 ml/min (tank, 60 psig; rotameter, 2); scavenger (air) flow rate, 250 ml/min (tank, 18 psig); hydrogen flow rate, 120 ml/min (tank, 16 psig).

Preparation of Standard Calibration Curves

One millimole of each of the 20 common amino acids was placed in a volumetric flask (1000 ml) along with hydrochloric acid (6 *N*). The volume of the resultant solution was adjusted to 1000 ml with the same acid. Samples (0.25, 0.50, 0.75, 1.0, 2.0, 3.0, and 4.0 ml) of this solution were placed in seven culture tubes (Pyrex 9826; screw top; Teflon-lined caps) and each of these solutions was evaporated (100 °C; oil bath) with the aid of a stream of dry nitrogen. Traces of water were removed by azeotropic distillation using anhydrous methylene chloride (150 µl). Evaporation (70 °C; oil bath) was facilitated by use of a stream of dry nitrogen. This process was performed three times. The amino acids in the dried mixtures were then converted to their *N*-trifluoroacetyl-*n*-butyl amino acid esters by the "direct esterification" method of Roach and Gehrke (36, 40). The samples were stored at −15 °C until they were analyzed. For gas chromatographic analysis, excess acylating reagent was removed (40 °C) from the same tubes with the aid of a stream of dry nitrogen. Anhydrous methylene chloride (100 µl) was then added to the dry *N*-trifluoroacetyl-*n*-butyl esters and aliquots (0.5–1.0 µl) were injected into the gas chromatograph.

The ratios formed by dividing the peak area (AAA) of each amino acid by the peak area (AIS) of the internal standard were calculated. A standard curve using the method of least squares was constructed for each amino acid by plotting the ratio obtained for that amino acid in each of the seven tubes, against the corresponding amount of the amino acid in the same tube. Arginine, histidine, and cystine could not be satisfactorily analyzed on the columns that were employed in the present study.

Preparation and Analysis of Samples

The procedure used for protein hydrolysis was essentially that described by Keutmann and Potts (21). The protein (10 mg) was placed in a clean, dry test tube (13 ×

TABLE 3. Amino acid composition (g/100 g) of casein isolated from the milks of different species

	Cow	Horse	Pig	Reindeer[a]			Caribou[b]		Moose[a]				Polar bear[a]			Fin whale[b]	
				Animal No. 1	Animal No. 2	Animal No. 3	Animal No. 1	Animal No. 2	Animal No. 1	Anaiml No. 2	Animal No. 3	Harp seal	Musk-ox	Animal No. 1	Animal No. 2	Dall sheep	Animal No. 1	Animal No. 2
Nitrogen, %	15.1	15.9	15.4	15.3	16.2	15.2	12.7	11.5	14.6	15.3	15.6	16.0	13.7	11.5	12.5	11.0	9.58	15.9
Amino acid composition, g/100 g																		
Alanine	3.79	3.40	3.61	2.71	2.43	2.89	2.83	2.58	2.52	2.38	2.45	2.69	3.23	2.23	2.48	2.47	2.50	3.00
Aspartic acid	8.82	7.43	8.11	6.93	7.85	7.11	7.82	7.06	6.07	7.38	7.36	9.71	8.03	8.55	8.20	6.19	5.64	7.28
Cysteine	0.00	0.10	0.25	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.18	0.00	0.80	0.00	0.00
Glutamic acid	26.7	27.0	27.8	26.5	25.7	25.8	25.4	24.3	19.7	23.5	23.1	25.3	21.5	24.6	24.7	15.5	13.9	16.3
Glycine	2.52	6.67	1.43	2.19	2.14	2.20	2.01	2.01	1.77	1.77	1.69	0.63	1.52	0.72	0.55	1.08	1.25	1.94
Hydroxyproline	0.00	0.05[c]	1.00[c]	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.13[c]	0.00	0.00	0.06[c]	0.00	0.00
Isoleucine	6.97	5.38	5.89	4.81	4.20	4.54	4.10	4.38	3.83	3.88	4.05	3.54	3.76	4.41	4.23	2.90	2.80	3.30
Leucine	13.5	11.0	10.5	10.4	9.72	10.1	9.29	9.28	8.31	8.55	8.79	10.7	9.11	9.19	9.44	5.14	5.69	6.60
Lysine	8.73	7.23	9.60	7.88	8.80	8.07	9.07	9.28	9.71	9.06	9.30	6.29	6.77	5.36	6.32	2.33	5.23	7.57
Methionine	3.26	1.61	2.42	2.83	2.71	2.71	2.17	2.16	1.91	2.08	2.40	0.87	2.03	1.22	1.32	0.75	1.08	1.11
Phenylalanine	5.94	4.28	5.04	4.64	4.62	4.70	4.24	4.02	3.50	4.18	4.07	3.47	3.93	3.89	3.85	2.04	2.47	3.20
Proline	15.4	12.2	12.6	9.43	11.5	11.4	9.40	9.97	7.94	8.74	9.21	8.81	9.78	10.1	10.9	3.97	3.79	4.95
Serine	10.1	8.81	8.56	11.8	11.2	13.5	12.0	10.9	6.63	9.16	10.2	7.91	8.98	9.28	9.57	6.19	6.48	6.31
Threonine	5.20	3.67	4.75	4.74	4.11	4.81	4.62	4.48	3.45	3.73	3.98	2.72	3.62	3.75	3.63	1.76	2.52	3.39
Tyrosine	4.42	4.38	5.59	6.88	6.26	6.97	5.59	5.87	4.57	6.30	6.52	5.87	7.08	4.59	5.13	5.34	3.05	3.20
Tryptophan	1.45	0.87	1.38	1.37	1.47	1.61	1.52	1.48	1.64	1.95	1.97	2.00	1.85	1.45	—	—	1.73	—
Valine	8.89	6.90	6.58	6.22	5.93	6.14	5.59	5.41	5.32	5.27	5.27	4.46	5.23	5.04	5.34	3.06	3.38	4.17

[a]Casein samples isolated from the milks of three different animals.
[b]Casein samples isolated from the milks of two different animals.
[c]The authors are aware that hydroxyproline has not been shown to occur in casein. Nevertheless peaks having the retention time of *n*-butyl-N-trifluoroacetyl hydroxyproline occurred on the chromatograms. No attempt was made to confirm the presence of hydroxyproline.

150 mm) along with constant-boiling HCl (5.7 *N*; 1.0 ml) containing mercaptoethanol (1:2000 v/v). The tube was then placed in a glass desiccator (diameter, 15 cm) that contained the same mixture of constant-boiling HCl and mercaptoethanol that was used for hydrolysis. The level of liquid in the desiccator was adjusted to the same level as that in the tube. The size of the desiccator made possible the hydrolysis of 50 samples at one time. The cover of the desiccator was held in place by means of a clamp. The desiccator was evacuated and then flushed with nitrogen. This procedure was repeated three times. After a final evacuation, the desiccator was placed in a forced-draft oven (110 °C) for 22 h. The tubes were cooled and opened and the hydrolysates were extracted with two portions (1 ml each) of diethyl ether to remove fatty acids. The samples were then dried under vacuum in a desiccator containing sodium hydroxide flakes. The drying process took about 8 h.

Distilled water (1 ml) was added to each tube to dissolve the dried hydrolysates. The contents of each tube were transferred quantitatively to separate Dowex 50X 12 (H^+ form) columns (1.25 × 1.25 cm). Distilled water (four portions, 1 ml each) was used to ensure a quantitative transfer from the tube to the column. The columns were washed with five portions (1 ml, each) of distilled water to remove sugars and carboxylic acids, and the washings were discarded. A volumetric flask (10 ml) was placed under each column and the column was washed with five portions (1 ml, each) of ammonium hydroxide (10% w/v) followed by 1-ml portions of distilled water until the effluent volume reached the mark.

Aliquots (2.0 ml) from each volumetric flask were placed in separate culture tubes (Pyrex 9826) and were evaporated to dryness (100 °C) with the aid of a stream of dry nitrogen. Traces of water were removed by azeotropic distillation using anhydrous methylene chloride. The samples were then subjected to the procedure of Roach and Gehrke (36, 40), as described for the standard mixtures. The chromatographic peak areas were determined for the individual amino acids in each sample. The ratio of the peak area for each amino acid (AAA) to that of *n*-butyl stearate (AIS) in each chromatogram was determined. From the ratios AAA:AIS, the number of micrograms of each amino acid present in each tube was determined by reference to the standard calibration curves. Tryptophan was determined by the method of Shaw and McFarlane (38). Figure 1 shows a typical gas chromatogram obtained with α-casein.

Results and Discussion

The results of the amino acid analysis of the caseins isolated from the milks of different species are given in Table 3. It will be noted that horse casein contained relatively high levels of glycine, and harp seal and polar bear casein contained relatively low levels of this amino acid. Fin whale casein contained much lower levels of glutamic acid than did the other casein preparations. The average values for the amino acid composition of reindeer casein were in close agreement with those reported by Luhtala *et al.*

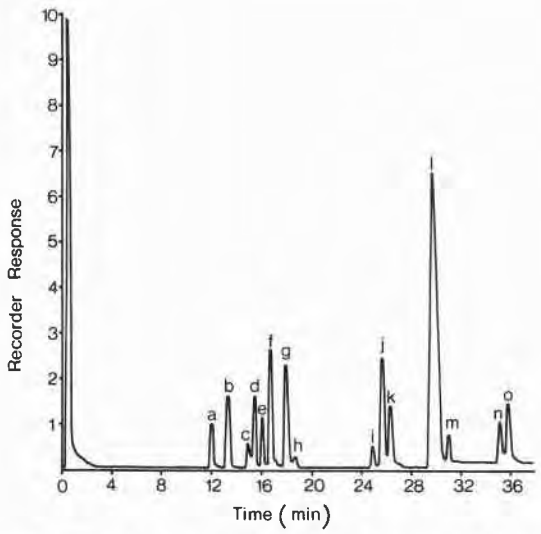

FIG. 1. Gas chromatogram of *N*-trifluoroacetyl *n*-butyl amino acid esters of α-casein hydrolysate. The individual peaks are identified as follows: *a*, alanine; *b*, valine; *c*, glycine; *d*, isoleucine; *e*, threonine; *f*, leucine; *g*, proline; *h*, serine; *i*, methionine; *j*, aspartic acid; *k*, phenylalanine; *l*, glutamic acid; *m*, tyrosine; *n*, *n*-butyl stearate (internal standard); *o*, lysine.

(29) for reindeer "protein," with the exception of serine. Harp seal casein contained less than half as much glycine and methionine, over three times as much serine, and about 60% more glutamic acid than did the casein of Northern fur seal (3). The amino acid composition of reindeer and caribou caseins were closely similar, as were the electrophoretic patterns of their caseins (25, 26). Since the electrophoretic properties of casein at pH 8.6 are undoubtedly influenced by the concentrations of aspartic and glutamic acids, it was not surprising to find that these two caseins, which have about the same concentrations of these amino acids, have similar electrophoretic mobilities (25). It should be stated, however, that no attempt was made to determine the amide content of the amino acid hydrolysates of the caseins isolated from the milks of these two species. Hence, it is not known whether the ratios of the free carboxyl groups to free amino groups in the casein hydrolysates from the two species were similar. Similarities in amino acid composition and electrophoretic mobility are to be expected in view of the fact that reindeer and caribou are assigned to the same species (*Rangifer tarandus*).

Histidine, arginine, and cystine have not been determined and hence it was impossible to calculate the limiting amino acids of the various caseins by chemical scoring techniques (6, 32).

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