A STUDY OF BACTERIOLOGICAL MEDIA: THE EXAMINATION OF CASAMIN E*

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Three batches of Casamin E have been examined quantitatively for their free and total amino acid content. Variation occurs in the free amino acid content in the three batches examined. The peptides were examined qualitatively. The batches were examined for their strepogenin activity using the media of Steele, Sauberlich, Reynolds and Baumann[1] and Kodicek and Mistry[2]. The stimulatory pattern appeared to be different with the two basal media.

In a previous paper[3] the quantitative examination of the amino acids in Difco Bacto-Casitone was reported. This paper describes the quantitative estimation of the free and total amino acids in three batches of Casamin E which is a pancreatic digest of casein manufactured by Mann Research Laboratories. The qualitative examination of the constituent peptides is given. The three batches were examined for their strepogenin activity.

Experimental

Quantitative estimation of the free amino acids. Three batches of casamin E designated A, B and C were examined. 0·1 g. of casamin was reacted with fluorodinitrobenzene (FDNB) at pH 9 and 40° for 1½ hours in 0·1 N potassium chloride solution with vigorous stirring. Excess FDNB was extracted with ether, after acidification the dinitrophenyl (DNP) amino acids and peptides were extracted with ether, a part remained suspended in the ether layer. This was removed by dissolving in acetone and adding it to the washed ether extract. The ether extract was evaporated to dryness under vacuum then subjected to the cold finger condenser to remove most of the dinitrophenol. The residue, as well as that from the aqueous extract remaining after ether extraction of the DNP-amino acids, was subjected to the quantitative paper chromatography technique of Levy[4] using the ethyl benzene system[5] in the first direction followed by 1·5 M phosphate buffer as previously described[3].

The total amino acids were estimated by hydrolysing 0·1 g. of casamin and reacting the hydrolysate with FDNB and then subjecting the DNP-amino acids to quantitative paper chromatography. The amino acid content of casein was determined by the same method and results were computed for a nitrogen content of 12·5 per cent. The results are given

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in Table I which shows in column 9 the amino acid content of casein obtained from the literature[6–15]. Results obtained by Gordon and others[16] are included in column 10. Results in columns 9 and 10 have been computed for a nitrogen content of 12·5 per cent.

TABLE I

THE QUANTITATIVE ESTIMATION OF THE FREE AND TOTAL AMINO ACIDS IN CASAMIN E

Amino acid	Free amino acid g./100 g.			Total amino acid g./100 g.					
							Casein		
	A	B	C	A	B	C	Determined	Literature*	Gordon and others[16]
Gly ..	0·191	0·16	0·38	1·61	1·64	1·76	1·56	1·52 (6)	2·16
Ala ..	0·643	0·605	1·58	2·56	2·70	2·69	2·59	2·8 (7)	2·40
Val ..	1·63	1·51	3·43	5·9	6·16	6·35	6·0	5·75 (8)	5·75
Leu's ..	6·45	5·90	9·25	12·35	13·23	13·32	12·85	14·3 (8)	12·25
Ser ..	0·98	1·0	1·81	4·92	4·90	5·01	3·92	4·72 (9)	5·03
Thr ..	0·85	0·833	1·78	3·57	3·83	3·71	3·85	3·60 (9)	3·92
Tyr ..	0·642	0·353	0·743	1·29	1·51	1·47	4·75	4·87 (10)	5·03
Phe ..	2·97	2·68	3·47	4·3	4·62	4·62	4·55	4·4 (8)	4·0
Met ..	1·37	1·33	2·36	2·47	2·33	2·47	2·3	2·48 (11)	2·24
Arg ..	1·90	1·99	2·49	2·5	2·85	2·70	3·4	3·2 (12)	3·28
His ..	0·92	0·733	1·23	2·34	2·25	2·14	2·46	2·56 (12)	2·48
Cys ..	—	—	—	0·242	0·242	0·254	0·164	0·272 (11)	0·272
Lys ..	4·2	4·0	4·73	6·1	6·25	6·25	6·97	6·55 (13)	6·55
Pro ..	0·13	0·12	1·1	8·6	8·9	8·5	8·23	9·27 (8)	9·05
Asp ..	1·46	2·35	6·4	23·6	23·4	23·9	23·4	23·4 (14, 15)	23·6
Glu ..	1·26								
Try ..	0·84	0·772	1·08	—	—	—	—	—	—
Total ..	26·436	24·286	41·833						
Nitrogen per cent	12·45	12·33	12·5						

* Number in parentheses refers to reference number.

Figure 1 shows the chromatogram of the DNP-amino acids and peptides of batch A, similar chromatograms were obtained with batches B and C. Three spots correspond to peptides and a fourth trailing spot in the aqueous extract is present. 0·2 ml. of the ether extract was applied on each of four sheets of Whatman 3 MM paper and the papers were subjected to two-dimensional chromatography. 0·2 ml. of the aqueous extract was chromatographed using the ethyl benzene system. The spots corresponding to the peptides were excised and eluted with water. The eluates from corresponding spots in the three batches were pooled together and then concentrated under vacuum. The solution was acidified with 6 N hydrochloric acid and the DNP-peptides were extracted with ethyl acetate to free them from salts and the extract evaporated to dryness under vacuum. The residue was dissolved in 0·3 ml. of 6 N hydrochloric acid and 0·15 ml. of the solution was completely hydrolysed at 105° for 24 hours. An aliquot was subjected to two dimensional chromatography for amino acid analysis[17]. The remainder of the solution was hydrolysed for 8 hours at 105° and an aliquot was examined for the *N*-terminal amino acid as the DNP-derivative by two dimensional chromatography[5]. All the peptides spots gave several DNP-amino acids thus indicating that they consist of a mixture of peptides. For example spot I of batch A gave aspartic* and glutamic* acids, trace of threonine, alanine*, valine*, leucines*,

proline, serine*, glycine, phenylalanine* and a trace of arginine. Lysine and the amino acids marked with an asterisk appeared as *N*-terminal residues.

Bacteriological examination. Three batches were tested for their strepogenin activity on *Lactobacillus casei* ATCC (7469) using the media of Steele and others[1] and Kodicek and Mistry[2]. The procedure followed for the culture and inocula was as that described by Ågren[18]. The

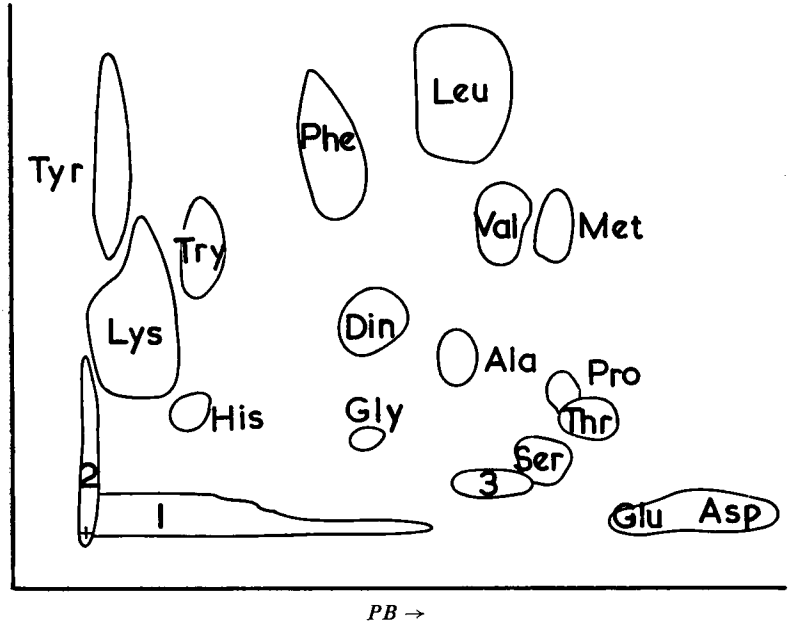

FIG. 1. Two dimensional chromatogram of DNP-amino acids and peptides in Casamin E, batch A. + point of application. *E*=direction of ethyl benzene developer. *PB*=direction of 1·5M phosphate buffer. *Din*=dinitrophenol.

method of assay was as previously reported[3]. The growth stimulatory effect was given as scale reading on the Klett-Summerson photoelectric colorimeter measured after 24, 48 and 72 hours incubation. The effect on lactic acid production was measured by titrating with 0·077 N sodium hydroxide using bromothymol blue as indicator. Results are given in Table II.

DISCUSSION

Table I shows that the content of free amino acids in the three batches of casamin varied from 24 to 42 per cent. While the difference is small between batches A and B, batch C shows more than 60 per cent increase in the total free amino acids over either A or B. The three batches of casamin show a higher content of free amino acids than the three batches of bacto-casitone previously examined[3], in which variation was from 15·9 to 17·5 per cent. This difference probably originates in the conditions of the enzymatic digestion of casein during the manufacturing

process. The free amino acids are not liberated in proportion to their occurance in the protein. The total amino acids were similar in the three batches and with the exception of tyrosine compared favourably with the reported data of casein and also with the values obtained by the quantitative paper chromatography technique. The uncorrected value for serine in column 8 was lower than that reported in the literature. This may result from the greater lability to acid hydrolysis of serine combined as phosphoserine in phosphoproteins compared with that of serine itself. It seems that some destruction of tyrosine takes place during the manufacturing process as evidenced by the low values of tyrosine in the three batches. From Figure 1 and *N*-terminal analysis of the peptide spots it is concluded that they consist of a mixture of peptides. There appears to be some variation in the peptides in corresponding spots in the three batches.

TABLE II

THE EFFECT OF CASAMIN E ON THE GROWTH OF *L. casei*

Time hours	Control	Casamin A mg. 0·1	0·5	1·0	Casamin B mg. 0·1	0·5	1·0	Casamin C mg. 0·1	0·5	1·0	Wilson's Liver L 0·1	0·5	1·0
					A—Medium of Steele and Others*								
24	7	53	114	145	45	92	117	24	59	84	5	15	23
48	17	131	239	287	119	235	286	75	201	235	20	56	88
72	256	164	255	300	163	260	305	128	237	273	70	118	137
					Acid Production†								
48	0	1·7	4·15	5·75	1·45	3·6	4·2	1·0	3·15	4·25	0·15	0·35	1·1
72	5·25	4·15	8·6	9·50	4·15	8·45	9·7	2·95	7·3	8·8	0·95	1·4	3·1
					B—Medium of Kodicek and Mistry*								
24	81	85	150	178	87	147	177	75	155	171	66	103	148
48	267	278	297	300	288	296	307	280	298	310	278	286	298

* Scale reading on the Klett-Summerson colorimeter. † ml. 0·077 N sodium hydroxide.

The three batches of casamin showed a stimulatory effect on the growth and the lactic acid production of *L. casei*. The pattern of stimulation differed with the basal medium used. Thus using the medium of Steele and others the stimulatory effect of casamin continued even after 72 hours. The growth stimulatory effect of batch C appears to be about 10 per cent less than that of batch A and B, whether this decrease is significant can not be decided from the data presented. The tubes containing 0·1 mg. casamin as well as those containing Wilson's liver fraction L showed less growth than the control after 72 hours incubation. Using the medium of Kodicek and Mistry[2] it was found that after 48 hours, the tubes containing all levels of casamin showed comparable growth.

The three batches of casamin showed comparable strepogenin activity with the three batches of Bacto-Casitone previously examined[3], although the free amino acid content differed. It seems that the strepogenin activity is exhibited by peptides of widely different structure.

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REFERENCES

1. Steele, Sauberlich, Reynolds and Baumann, *J. biol. Chem.*, 1949, **177**, 533.
2. Kodicek and Mistry, *Biochem. J.*, 1952, **51**, 108.
3. Habeeb, *J. Pharm. Pharmacol.*, 1959, **11**, 157.
4. Levy, *Nature, Lond.*, 1954, **174**, 126.
5. Habeeb, *J. Pharm. Pharmacol.*, 1958, **10**, 591.
6. Shankman, Camien and Dunn, *J. biol. Chem.*, 1947, **168**, 51.
7. Tristram, *Biochem. J.*, 1946, **40**, 721.
8. Henderson and Snell, *J. biol. Chem.*, 1948, **172**, 15.
9. Rees, *Biochem. J.*, 1946, **40**, 632.
10. Lugg, *ibid.*, 1938, **32**, 2123.
11. Kassell and Brand, *J. biol. Chem.*, 1938, **125**, 435.
12. Macpherson, *Biochem. J.*, 1946, **40**, 470.
13. Gale, *ibid.*, 1945, **39**, 46.
14. Hac and Snell, *J. biol. Chem.*, 1945, **159**, 291.
15. Bailey, Chibnall, Rees and Williams, *Biochem. J.*, 1943, **37**, 360.
16. Gordon, Semmett, Cable and Morris, *J. Amer. chem. Soc.*, 1949, **71**, 3293.
17. Habeeb and Shotton, *J. Pharm. Pharmacol.*, 1956, **8**, 197.
18. Ågren, *Acta physiol. scand.*, 1949, **17**, 55.