A STUDY OF BACTERIOLOGICAL MEDIA; THE EXAMINATION OF BACTO-CASITONE*

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Three batches of Bacto-Casitone have been examined quantitatively for their free and total amino acids using paper chromatography. Variation occurs in the free amino acids in the three batches. The spots due to the peptides have been found to be a mixture of peptides. When casitone was examined for its strepogenin activity using the medium of Steele, Sauberlich, Reynolds and Baumann[1], and Kodicek and Mistry[2] the stimulatory pattern in the two media appeared to be different. The three batches showed similar strepogenin activity.

PROTEIN hydrolysates by virtue of their content of amino acids and peptides are important ingredients of many microbiological culture media. In previous papers[3,4] the qualitative identification of the constituent amino acids and peptides in "Oxoid" bacteriological peptone was reported. This paper describes the quantitative estimation of the free and total amino acids in three batches of "Difco" Bacto-Casitone, together with a qualitative examination of the constituent peptides. The three batches were examined for their strepogenin activity.

EXPERIMENTAL

Quantitative Estimation of Free Amino Acids

Three batches of casitone designated A, B and C were used. 0·1 g. was dissolved in water and the pH was brought to 9 by 0·5N sodium hydroxide, the volume was adjusted to 15 ml., potassium chloride was added to give an 0·1N solution[5], then 0·5 ml. of fluorodinitrobenzene (FDNB) was added and the reaction was allowed to proceed at 40° for 1½ hours with vigorous stirring, using a magnetic stirrer, the pH was kept at 9 by careful addition of 0·5N sodium hydroxide. After the reaction excess FDNB was removed by extraction with ether. The solution was then acidified and extracted with ether. The combined ethereal extracts were washed with water to which a few drops of 6N hydrochloric acid were added. The ether extract was evaporated under vacuum to dryness and the residue was subjected to the cold finger condenser[6] to remove most of the dinitrophenol. The residue was dissolved in 3 ml. of methanol-methylethyl ketone 1:1 (v/v). The aqueous extract was evaporated to dryness and subjected to column chromatography on talc-celite to remove salts[7]. The column was extruded and the coloured zone

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was eluted with ethanol-6N hydrochloric acid 4:1 (v/v). The eluate was evaporated to dryness and the residue was dissolved in 3 ml. ethanol-6N hydrochloric acid. 0·05 ml. of the ether extract and 0·02 ml. of the aqueous extract were subjected to the quantitative paper chromatography technique of Levy[8], using the ethyl benzene system[9] in the first direction followed after drying the paper by 1·5M phosphate buffer in the second dimension. The excised spots of dinitrophenyl (DNP) amino acids were eluted with a suitable volume of 1 per cent sodium bicarbonate. The optical density reading was converted to the absolute value of the amino acid using the factors previously given[9].

Quantitative Estimation of Total Amino Acid.

0·1 g. of casitone was completely hydrolysed by refluxing with 10 ml. 6N hydrochloric acid for 24 hours after which the solution was evaporated to dryness in a vacuum desiccator over sodium hydroxide. The residue was taken up in water and subjected to the reaction and extraction procedures outlined above. The residue from the ether was dissolved in 2 ml. methanol-methylethyl ketone and that from the aqueous extract in 2 ml. ethanol-6N hydrochloric acid, 0·015 ml. of each was subjected to quantitative paper chromatography. The reaction was carried out in duplicate and paper chromatography in triplicate. The results are given in Table I for the three batches. Figure 1 shows the chromatogram of

TABLE I

THE QUANTITATIVE ESTIMATION OF THE FREE AND TOTAL AMINO ACIDS IN BACTO CASITONE

Amino acid	Free amino acids, g./100 g.			Total amino acid, g./100 g.		
	A	B	C	A	B	C
Gly	0·226	0·197	0·224	1·97	2·03	2·11
Ala	0·513	0·614	0·62	2·97	3·01	3·02
Val	0·802	0·82	0·893	6·25	6·27	5·97
Leu's	4·43	4·57	5·0	13·75	13·3	13·65
Ser	0·635	0·557	0·612	4·25	4·23	4·03
Thr	0·521	0·497	0·574	3·17	3·58	3·57
Tyr	0·272	0·44	0·427	1·56	1·59	1·54
Phe	2·24	2·39	2·66	4·67	4·61	4·75
Met	0·53	0·568	0·72	2·50	2·47	2·36
Arg	1·78	1·62	1·58	2·5	2·55	2·42
His	0·209	0·274	0·293	1·83	2·02	2·33
Orn	0·037	0·082	0·071	0·07	0·09	0·048
CyS	—	—	—	0·288	0·287	0·30
Lys	3·11	1·7	1·92	6·23	6·33	6·22
Pro	0·226	0·202	0·225	8·49	8·60	8·50
Asp and Glu	1·32	0·89	1·22	23·6	24·2	23·9
Try	0·448	0·461	0·488	—	—	—
Total	17·288	15·882	17·528			

DNP amino acids and peptides obtained with batch B. Batch A and C gave similar pictures. It is seen that there exist 3 spots which do not correspond to the position of the amino acids. These disappear on hydrolysis. In addition, there is a trailing spot in the aqueous extract. 0·2 ml. of the ether extract was applied on Whatman 3MM paper and subjected to two dimensional chromatography; 0·2 ml. of the aqueous extract was subjected to chromatography using the ethyl benzene system. Four sheets were thus treated. The spots due to the peptides were eluted with acetone-ammonium hydroxide 4:1 (v/v). The eluate was evaporated

to dryness, the residue was dissolved in 0·2 ml. of 6N hydrochloric acid, and of this 0·1 ml. was hydrolysed in a sealed tube for 8 hours at 105°. After removal of the acid the residue was taken up in a few drops of acetone and an aliquot was subjected to two dimensional chromatography using the ethyl benzene system and 1·5M phosphate buffer to detect the *N*-terminal amino acid of the peptide as the DNP-derivative.

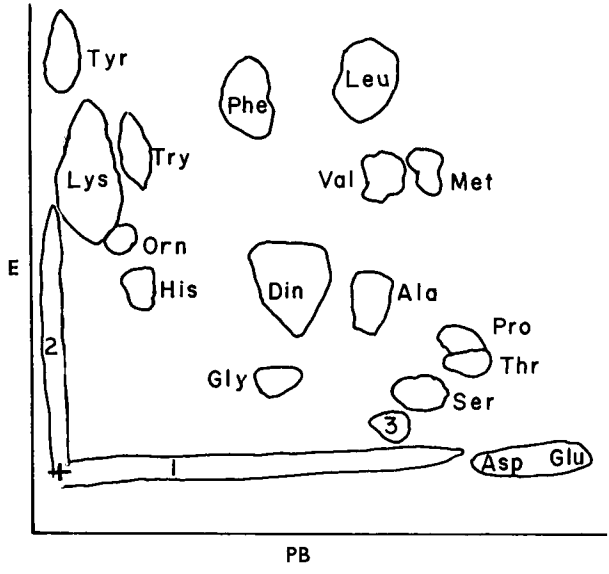

FIG. 1. Two-dimensional chromatogram of DNP-amino acids and peptides in bacto casitone, batch B. + Point of application, *E*, direction of ethyl benzene developer. *PB*, direction of 1·5M phosphate buffer developer. *Din*, dinitrophenol.

The second part of 0·1 ml. was completely hydrolysed for 24 hours at 105° and then after removal of the acid the residue was dissolved in a few drops of 10 per cent *iso*propanol. An aliquot was subjected to two dimensional chromatography using the butanol-acetic acid-water system 4:1:5 in the first direction followed after drying the paper, by spraying the paper with phosphate buffer pH 6·2 and developing in the second direction with phenol saturated with buffer[3]. The quantity of data resulting from this examination does not permit profitable tabulation, for example, in spot No. 1, which is typical, amino acids aspartic, serine*, threonine*, alanine*, glutamic*, valine*, methionine, leucines*, proline, glycine* and possibly histidine were present in the total hydrolysate; while arginine, lysine and those marked with an asterisk were identified as *N*-terminal amino acid residues indicating considerable heterogeneity in the spot.

Bacteriological Examination

Growth stimulating factors have been reported by various workers in enzymatic digests of various proteins. Woolley and others[8,9] found that

casein partially acid hydrolysed and tryptic digests of casein and other purified proteins stimulated the growth of *Lactobacillus casei*. These workers have given the name strepogenin to the growth factor(s) and their studies of the properties of strepogenin led them to suggest it had a peptide character. Merrifield and Woolley[12] have isolated a peptide serylhistidylleucylvalylglutamic acid from acid digested insulin and found that it has 80 units of strepogenin activity. Later Tritsch and Woolley[13] isolated the disulphide of leucylvalylcysteinylglycylglutamylarginine from an enzymic digest of insulin and showed it to have 200 units of strepogenin activity. Ågren[14] found that a commercial tryptic digest of casein stimulated the growth of *L. casei* when grown on the medium of Henderson and Snell[15]. Later[16], he isolated a peptide fraction from calf's plasma and found that it has a stimulating effect both on growth and lactic acid production of *L. casei* after 72 hours incubation when grown on the medium of Steele and others[1]. The three batches of casitone were tested for their stimulatory effect on *L. casei* ATCC (7469). The procedures followed for the culture and inoculum were as described by Ågren[14]. Assays were made in triplicate in 18 × 150 mm. Pyrex culture tubes and in a total volume of 5 ml. The casitone was tested at 0·1, 0·5 and 1·0 mg. concentration. The amount of casitone adjusted to pH 6·8 was added in a volume of 2·5 ml. to 2·5 ml. of the basal medium of Steele and others[15]. Tubes containing 0·1, 0·5 and 1·0 mg. of Wilson's liver fraction L were included for comparison. The tubes were autoclaved at 15 lb. for 5 minutes. A drop of uniform size of a diluted suspension of *L. casei* in saline was added to each tube by a syringe equipped with a needle, the end of which was ground flat. The growth stimulatory effect was given as scale reading on the Klett–Summerson photo-electric colorimeter, this was measured after 24, 48 and 72 hours. The effect on the lactic acid production was measured by titrating with 0·77N sodium hydroxide. The strepogenin activity was also determined on the medium of Kodicek and Mistry[16] in which the glucose content was reduced to 2·5 per cent. Results are given in Table II.

TABLE II

THE EFFECT OF CASITONE ON THE GROWTH OF *L. casei*

Hours	Control	Casitone A, mg.			Casitone B, mg.			Casitone C, mg.			Wilson's liver L, mg.		
		0·1	0·5	1·0	0·1	0·5	1·0	0·1	0·5	1·0	0·1	0·5	1·0
					A. Medium of Steele and others*								
24	2	36	96	109	28	70	90	22	60	95	9	21	30
48	13	96	238	271	92	213	256	93	206	261	28	78	123
72	250	146	271	315	142	253	303	140	247	305	—	143	180
					Acid Production†								
24	0	0·2	0·42	0·57	0·15	0·35	0·50	0·13	0·35	0·45	0·05	0·13	0·15
48	0	2·05	5·15	6·45	1·75	4·6	5·85	1·7	4·7	6·3	0·33	1·45	3·05
72	4·7	4·05	8·9	9·6	3·45	7·95	9·7	3·65	7·7	9·8	—	3·4	4·85
					B. Medium of Kodicek and Mistry*								
24	83	108	145	174	127	147	171	95	152	167	77	118	168
48	267	269	281	285	275	277	282	271	274	280	270	282	295

* Scale reading on the colorimeter. † Ml. of 0·077N alkali.

Discussion

From Table I it is seen that some variation occurs in the free amino acids among the three batches and that the amino acids are liberated to varying degrees. Arginine, phenylalanine, leucines and lysine occur in a higher proportion relative to their total occurrence in casitone than proline, aspartic and glutamic acids which seem to be liberated slowly. This appears to be due to variation in the susceptibility of peptide bonds involving these amino acids. The variation in the free amino acids may be due either to some variation in the purity of pancreatic extract used for the digestion of casein or some variation in the conditions during the digestion. Harding and MacLean[17] subjected casein to the pancreatic enzymes and followed the hydrolysis by the determination of the α-amino acid nitrogen. They found that even at the end of 216 hours the hydrolysis of casein was still proceeding. Ornithine is detected and it seems likely that it occurs only in the free state, it may be produced from arginine during the digestion.

Figure 1 and the results of analysis of the spots, a typical example of which was given in the experimental section, show that the spots due to the peptides consist of a mixture of peptides. This mixture of DNP peptides proved to be difficult to separate either by paper electrophoresis[18] or paper chromatography. There seems to be some variation in the constituent peptides in corresponding spots in the three batches.

It is observed from Table II that casitone has a stimulatory effect on the growth and lactic acid production of *L. casei* when grown on the medium of Steele and others. The stimulatory effect lasts as long as 72 hours. The results are at variance with those recorded by Woolley, Ågren and Kodicek and Mistry for strepogenin where the stimulatory effect is detected after 18 to 24 hours and where after 48 hours all tubes whether supplemented or not showed maximal growth and acid production. That this variation is due to the basal medium used in the assay is evidenced by the results obtained when the basal medium of Kodicek and Mistry is used; the stimulation is seen after a 24 hour incubation while after 48 hours all tubes including the controls showed similar growth. The medium of Steele and others supported little if any growth after 48 hours, this was similar to results obtained by Ågren[16], but his conclusion that the peptide fraction isolated from calf's plasma behaved differently from strepogenin may have been different had he tested for the stimulatory effect on a medium that is used for strepogenin or included in his assay a material with known strepogenin activity for comparison. Tubes containing Wilson's liver fraction L as well as casitone at 0·1 mg. level showed less growth after 72 hours than the controls. The three batches of casitone although they showed some variation in their constituent peptides yet showed similar strepogenin activity. As peptides may have varying strepogenin activity, it seems that strepogenin active peptides are similarly liberated in the three batches.

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