ACTA CHEMICA SCANDINAVICA 6 (1952) 999–1005

On the Microbiological Activity of a Plasma-peptide Fraction

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The present paper describes the microbiological activity of a peptide fraction prepared from calf's plasma by means of heat coagulation and precipitation with picric acid. The preparation is free from the plasma muco-protein recently described by Winzler *et al.*[1] and contains no free amino acids. Other characteristics will be published elsewhere.

MICROBIOLOGICAL PROCEDURES

The procedures followed for the cultures and inoculum have been described in previous papers [2,3]. Assays were carried out in 18 × 150 mm Pyrex culture tubes and in a total volume of 5 ml. The peptide solution, adjusted to pH 6.8, was added in volumes of 2.5 ml to 2.5 ml of the basal medium of Steele *et al.*[4] For *L. citrovorum* (8081) the addition of 0.4 ml of a concentrated liver preparation (Heptomin) to 100 ml of synthetic medium was used [5]. *L. lactis* (8000) was grown on the medium given by Shorb [6], and *L. leichmanii* was cultured on the medium described by Capps *et al.*[7]

RESULTS

The growth was followed simultaneously by photoelectric readings and by titrations. Typical results are given in Table 1 and 2. The figures in Table 1 clearly show the general growth stimulating effect of the peptide preparation. With the exceptions of *S.faecalis* R (8043) and *S.lactis* (9790) the other tested lactobacilli are more or less stimulated by the addition of the plasma peptides to the medium. For three microorganisms, *L.mesenteroides*, *L.fermenti* 36, and *L.arabinosus*, the effect is obvious only in the first 12 hours of incubation. For two of the other lactobacilli, *L.casei* and *L.delbrückii* 3, the effect is still observable after 72 hours of incubation. *L.mesenteroides* P-60, one of the microorganisms most commonly used for microbiological amino acid determination, is not stimulated by the presence of plasma peptides in the medium when an incubation time of 24 hours or longer is used.

Table 1. Growth of lactobacilli in synthetic media supplemented with two concentrations of a plasma-peptide preparation.

	Growth after*											
	12 hours			24 hours			48 hours			72 hours		
	control	1 mg peptide	0.1 mg peptide	control	1 mg peptide	0.1 mg peptide	control	1 mg peptide	0.1 mg peptide	control	1 mg peptide	0.1 mg peptide
L. citrovorum (8081)	10	28	21	20	37	31	26	41	34	40	58	48
L. citrovorum (8082)	5	28	17	125	160	148	210	203	195	200	190	195
L. casei (7469)	1	13	5	6	141	30	34	340	152	214	338	218
S. faecalis R (8043)	114	125	122	228	220	219	254	242	255	240	224	226
L. mesenteroides P 60 (8042)	90	181	167	280	288	292	310	320	320	300	318	317
L. fermenti 36 (9338)	9	56	42	123	154	130	196	180	170	194	180	165
S. lactis (9790)	150	164	150	228	236	235	242	250	245	220	232	222
L. delbrückii 5 (9595)	0	12	4	5	58	20	230	200	190	238	250	210
L. delbrückii 3	2	10	9	37	60	20	202	297	215	230	300	231
L. arabinosus (8014)	116	222	206	405	420	410	475	468	469	450	450	450

* Given as scale readings on the Klett-Summerson photoelectric colorimeter. The reading O corresponds to 100 per cent transmission and the values have been corrected for the blank values.

The effect on the lactic acid production is shown in Table 2. It is of considerable biological interest to note that the plasma peptide fraction has a stimulating effect both on growth and on lactic acid production after 72 hours of incubation. The most conspicuous results were obtained with *L.casei*, *L. delbrückii* 5, and *L.delbrückii* 3. The mechanism by which the lactic acid production is stimulated will be further investigated.

The growth stimulating effect recorded here both by photoelectric readings and titrations differs in some ways from that of the strepogenin effect found by Woolley [8]. He used *L.casei* and *S.lactis* as test organisms. Neither of the two types of *Streptoccus lactis* used in this investigation were stimulated by the addition of the plasma peptide fraction to the medium. With strepogenin present in the medium Woolley recorded a maximal growth effect after 18 hours of incubation. After 72 hours all tubes, whether supplemented or not, showed maximal growth and acid production. Similar results were obtained by the present author in microbiological tests with proteolysed liver and casein extracts [3]. When the plasma peptide fraction is added to the basal medium, however, the lactic acid production is stimulated even when an incubation time of 72 hours is used.

Table 2. Lactic acid production in synthetic media supplemented with two concentrations of a plasma peptide preparation.

Organism	ml of acid produced after 72 hours of incubation *		
	Control	1 mg peptide	0.1 mg peptide
L. citrovorum 8081)	1.4	2.4	1.8
L. citrovorum (8082)	5.8	6.4	5.8
L. casei (7469)	5.8	16.4	9.8
S. faecalis R (8043)	4.4	4.6	4.4
L. mesenteroides P 60 (8042)	14.8	16.8	15.4
L. fermenti 36 (9338)	6.8	8.6	7.4
S. lactis (9790)	5.0	6.0	5.2
L. delbrückii LD 5 (9595)	4.4	11.0	7.8
L. delbrückii LD 3	11.2	17.5	13.1
L. arabinosus (8014)	18.6	20.1	20.0

* Given as ml of 0.05 *N* NaOH to titrate 5 ml of final solution. The values are mean values from three tubes and have been corrected for the blank titrations.

With this difference in mind it was of special interest to investigate the growth effect of the plasma peptides on two other test organisms, *L.lactis* (8 000) and *L.leichmannii* (4797) used for determinations of vitamin B_{12}. Since Bird and Hoevet [9] have recently shown that this vitamin can be bound to a variety of proteins, the possibility could not be excluded that the peptide fraction contained some bound vitamin in an available form. To determine whether or not this was actually the case, experiments were carried out as parallels to those described in Table 1 and 2. The results are given in Table 3. From the figures indicating lactic acid production by the two microorganisms it seems probable that there is no available vitamin B_{12} present in the peptide preparation. The same conclusion can be drawn from the photoelectrice readings obtained with L.lactis. On the other hand, some of the results seem to suggest that the peptide fraction can have a synergistic effect with vitamin B_{12}. The growth figures obtained with *L.citrovorum* (8081) in Table 1 and 2 and in other experiments do not exclude the possibility that the stimulating effect of the peptide fraction to some extent may be caused by the citrovorum factors [10,3] or other unknown growth factors.

The question whether amino acids in peptide linkages can be utilized by lactobacilli or not has previously been investigated by means of synthetic peptides [11,12]. It was shown that all of the bacteria listed in Table 1—3 of the

Table 3. Growth of L. lactis (8000) and L. leichmannii (4797) on synthetic media supplemented with submaximal amounts of vitamin B_{12} and two concentrations of a plasma peptide preparation.

Organism	Growth after[1]							
	12 hours		24 hours		48 hours		72 hours	
	1 mg peptide	0.1 mg peptide	1 mg peptide	0.1 mg peptide	1 mg peptide	0.1 mg peptide	1 mg peptide	0.1 mg peptide
L. lactis + B_{12}[2])	1	2	3	5	2	3	84	84
L. lactis	4	1	6	5	5	6	40	40
L. leichmannii + B_{12}[2])	18	9	21	8	36	8	52	11
L. leichmannii	2	0	4	1	5	3	6	4
	Ml of acid produced in the same series[3]							
L. lactis + B_{12}[2])							2.4	2.6
L. lactis							0.4	0.4
L. leichmannii + B_{12}[2])							2.0	0.6
L. leichmannii							0.2	0

[1] Given as scale readings on the Klett-Summerson photoelectric colorimeter. The reading O corresponds to 100 per cent transmission and values have been corrected for blank values.

[2] Medium supplemented with 0.2 mμg of B_{12}. Maximal growth with 2.0 mμg of B_{12}.

[3] Given as ml of 0.05 *N* NaOH to titrate 5 ml of final medium. The values are the means from three tubes and have been corrected for blank values.

present investigation could utilize the leucine of several leucine containing tri- and dipeptides supplemented to the basal medium instead of the free amino acid. Virtanen and Nurmiko [13] recently demonstrated that *L.mesenteroides* P-60 could hydrolyze synthetic glycine and leucine peptides replacing these amino acids in the medium. How the peptides of deproteinized plasma behave in this respect has previously not been investigated. Henderson *et al.*[14] believe that the activity of such a proteinlike fraction is probably not available to lactobacilli since it is non-dialysable. From the methodological point of view it was of interest to analyze the availability of our peptide fraction.

L.mesenteroides P-60 is one of the lactobacilli most commonly used for microbiological amino acid determinations. The ability of that microorganism to use the hydrolyzed and unhydrolyzed peptide fraction supplemented to the basal medium instead of different free amino acids was investigated. Ten of the amino acids known by paperchromatography to be present in the hydrolysate were analyzed according to previously described methods. [15,16]

The experiments clearly showed that *L.mesenteroides* could not hydrolyze or utilize the amino acids of the plasma peptide when it replaced any of the ten free amino acids excluded from the synthetic medium. From the analytical data of the peptide hydrolysate it was calculated that the amino acids composition of the peptide fraction was the following

Arginine	10.9 %	Lysine	7.9 %
Glutamic acid	5.1 »	Methionine	0.8 »
Histidine	4.8 »	Phenylalanine	4.4 »
Isoleucine	4.0 »	Threonine	5.1 »
Leucine	8.0 »	Valine	5.2 »

The figures are not corrected for ash.

The results correspond to what is found in animal protein hydrolysates where the ten essential amino acids often correspond to about 50 per cent of the total amount. With regard to the discussed relationships to the glutamic acid-rich strepogenin the low glutamic acid content may be stressed. There is a comparatively high percentage of basic amino acids, *eg.* 23.6. In this respect the peptide fraction resembles the basic peptide, secretin, which is a heat stable polypeptide precipitated by picric acid and containing about 20 per cent of basic amino acids [17]. The possible requirements of basic peptides for the synthetis of nucleoproteins may also be mentioned in this connection.

Series of experiments were also carried out with *L.casei* and *L.delbrückii* 5 to determine if these microorganisms could utilize the plasma peptide when it replaced some amino acids excluded from the synthetic medium. As shown in Table 2 the lactic acid production of these two microorganisms was much more stimulated than in the case of *L.mesenteroides*. The results obtained with *L.casei* cultivated on the incomplete medium are given in Table 4. From a comparison with Table 2 it can be observed that the lactic acid production in the complete medium without peptide addition, is 5.8 ml as compared to 5.0—5.8 ml when the highest submaximal amount of the excluded amino acid, 40 μg, is added to the incomplete medium. The complete medium contains 310 μg of histidine, 6250 μg of leucine, and the same amount of valine per tube, and it is obvious that the tenfold increase of the submaximal amounts of amino acids does not effect the lactic acid production in the complete medium. Hence the effect of the peptides in the complete medium can not be due to the small amounts of free amino acids which the microorganisms possibly could set free from the peptide. The growth effect must in some way be connected with the action of the unhydrolyzed or partially split molecules.

The peptide fraction has some stimulating effect when added to the incomplete medium corresponding to 0.8—2.0 ml of acid per 0.1 mg of subs-

Table 4. Growth of L. casei in the medium of Steele et al[4] supplemented with the plasma peptide fractions instead of free amino acids. Incubation time 72 hours.

Amino acid omitted from the medium	Ml of acids produced with the following amounts of substances added per tube [1]					
	Free amino acid				Peptide	
Histidine	40 μg [2]	20	10	5	400 μg [3]	100
	5.0 ml	4.4	3.8	3.6	6.8	1.8
Leucine	40 μg [2]	20	10	5	400 μg	100
	5.8 ml	4.4	3.4	2.8	5.6	2.0
Valine	40 μg [2]	20	10	5	400 μg	100
	5.6 ml	3.8	2.6	1.8	2.2	0.8

[1] Given as ml 0.05 *N* NaOH to titrate 5 ml of final solution. The values are mean values from three tubes and have been corrected for blank titrations.

[2] The complete medium contains 310 μg of histidine, 6250 μg of leucine and 6250 μg of valine per 5 ml of final medium.

[3] 400 μg of peptide contains 19 μg of histidine, 32 μg of leucine, and 21 μg of valine.

tance. From Table 2 it can be observed that a similar amount added to the complete medium is accompanied by a lactic acid production which is from 5 to 10 times as large. From the figures in Table 4 it can not be definitely settled whether the effect of the peptide fraction in the incomplete medium depends on the action of the whole peptide molecules or is due to a cleavage to compensate for the excluded amino acid. However, it may be significant that the lactic acid produced by the peptides does not correspond to the amount which could be expected from the amino acid composition of the preparation. The most probable explanation of the low effect in the incomplete medium is that the excluded amino acids limit the growth.

The results obtained with *L.delbrückii* 5 were of the same qualitative nature as those obtained with *L.casei* recorded in Table 4. The conclusion drawn above that the growth stimulating effect must be ascribed to something else than the common amino acids present in the peptide molecules, is also substantiated by the experience gained in the tests with *L.delbrückii* 5.

The plasma peptide fraction is soluble in 80 per cent ethanol. Accordingly it may be present in "protein-free" amino acid extracts from plasma or organs prepared by this method. The presence of the peptides in such extracts may influence the microbiological amino acid determinations when *L.casei*, *L.delbrückii* 5 or LD 3 are used as tests organisms but not with *L.mesenteroides* P-60. Plasma filtrate deproteinized with picric acid or tungstic acid precipitation are free from the peptide fraction presently investigated.

SUMMARY

A plasma peptide fraction prepared from calf's plasma stimulates growth and lactic acid production of a series of lactobacilli, in some instances even when an incubation time of 72 hours is used. The fraction does not contain directly available vitamin B_{12}, but may contain citrovorum factors or other unknown growth factors. A microbiological amino acid analyses of the preparation shows a certain predominance of basic amino acids. The glutamic acid content is low.

The investigation was supported by a grant from Swedish Medical Research Council. The technical assistance of Mr. S. Eklund, Mr. E. Kristenson and Mrs. I. Kristenson is greatfully acknowledged.

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Received October 9, 1951.