ACTA CHEMICA SCANDINAVICA 2 (1948) 611—619

The Utilization of Peptide Bound Amino Acids by Lactic Acid Bacteria. II

GUNNAR ÅGREN

Department of Medical Chemistry, University of Uppsala, Sweden

Some of the reasons for studying the peptide utilization of lactic acid bacteria were given in a previous paper [1]. Briefly, it could be demonstrated that some lactic acid bacteria were capable of using the essential amino acids leucine and valine supplied to the basal medium in the form of synthetic peptides only when these amino acids were incorporated in certain orders in the peptides. It was also suggested that these investigations further developed could be useful in the identification of the order in which the amino acids of natural peptides are linked together. Similar results have subsequently been obtained by Krehl and Fruton [2]. These authors also suggested that differences in the effectiveness of several leucine peptides in replacing the free amino acid may be taken to reflect differences in the rate of cleavage of the peptides by bacterial peptidases. A direct comparison of the results obtained by Krehl and Fruton with those obtained by Ågren [1] were in some cases possible when the same bacteria, *Streptococcus faecalis* (9790) and the same peptides, leucyl-glycine and glycyl-leucine, were tested. According to Krehl and Fruton only 70 to 80 per cent of the bound leucine was available in these peptides while we found a 100 per cent utilization. These quantitative differences could possibly be explained by differences in the composition of the synthetic media used by the investigators. However, very little is known about the nutritional requirements of lactic acid bacteria with regard to essential amino acids supplied in peptide form to different basal media. In order to extend the basis for comparison the peptides previously studied [1] on the basal media of Stokes *et al.*[3] were now supplemented in the same way by to the recently described medium of Henderson and Snell [4]. For some of the lactic acid bacteria the medium was used in 0.5 times the concentration of all ingredients of the original Henderson and Snell medium with the exception of Salt C which was kept at the original concentration (*cf.* Ågren [5]).

EXPERIMENTAL

The microorganisms used were *Streptococcus faecalis* (9790),* *Lactobacillus delbrückii* LD5 (9595), *Leuconostoc mesenteroides* P 60 (8042), *Streptococcus faecalis* R (8043) *Lactobacillus casei* (7469), *Lactobacillus citrovorum* (8081), *Lactobacillus citrovorum* (8082), *Lactobacillus dextranicum* (8086), *Lactobacillus arabinosus* 17—5 (8014) and *Lactobacillus fermenti* 36 (9595). The assay technique of Henderson and Snell [4] was followed. Separate solutions of basal medium, standard amino acid, peptides and inocula were delivered to 6 inch test tubes. The final volume in each case was 6 ml. The standard and peptides were each run at three levels and from five to six tubes were employed at each level. The peptides described in the previous communication [1] were used and in

Table 1. Lactic acid production of Streptococcus faecalis (9790) and Streptococcus faecalis R (8043) in the medium of Henderson and Snell supplemented with leucine, tryptophane or tyrosine containing peptides. Incubation time 48 hours.

Peptides tested	*Streptococcus faecalis* (9790)				*Streptococcus faecalis* R (8043)					
	Amount of free or bound essential amino acid** per tube in γ									
	0	15	30	60	0	3	6	15	30	60
	ml***	ml	ml	ml	ml	ml	ml	ml	ml	ml
L-Leucine	1.6	5.0	8.4	10.8	0.5	—	—	3.3	5.4	8.8
DL-Alanyl-DL-leucyl-glycine	1.5	4.8	6.2	8.9	0.5	—	—	3.3	5.4	8.5
Glycyl-DL-leucyl-DL-alanine	1.8	2.1	2.2	2.1	0.6	—	—	0.8	0.8	0.8
Glycyl-DL-leucylglycine	1.6	4.9	7.8	9.9	0.5	—	—	3.4	5.3	8.9
DL-Leucylglycylglycine	1.5	5.0	7.6	9.9	0.5	—	—	3.1	5.7	8.6
DL-Alanyl-DL-leucine	1.5	3.6	5.8	8.6	0.5	—	—	2.5	4.1	6.7
DL-Leucyl-DL-alanine	1.6	3.6	5.1	6.9	0.6	—	—	1.5	1.6	3.7
Glycyl-DL-leucine	1.7	4.8	7.5	10.1	0.6	—	—	3.0	4.6	8.0
DL-Leucylglycine					0.7	—	—	3.2	5.3	8.5
L-Tryptophane					0	4.5	7.7	12.5	13.4	—
Glycyl-L-tryptophane					0	4.7	6.0	13.5	14.0	—
L-Tyrosine					5.8	—	—	7.9	9.4	12.5
Glycyl-L-tyrosine					5.8	—	—	8.9	10.2	11.1
	Complete medium 14.4 ml				Complete medium 14.0 m					

* All bacteria from the American type culture collection, Georgetown University, Washington, D. C.

** Peptides were added in amounts calculated to give the same levels of L-leucine, L-tryptophane or L-tyrosine as used in the corresponding standard curves.

*** Given as ml of 0.05 *N* NaOH to titrate 6 ml of final solution. The values are corrected for the blank titrations.

addition DL-leucyl-glycyl-glycine, glycyl-L-tryptopane and glycyl-L-tyrosine (Hoffmann-La Roche) were investigated. The standard amino acids were Merck preparations. The concentration levels of the tested peptides were as in the previous communication such that the amount of the essential amino acid of natural configuration they could yield fell within the range of the corresponding free amino acid amounts used in the standard curve.

RESULTS

The data obtained with *Streptococcus faecalis* (9790) and *Streptococcus faecalis* R (8043) are presented in Table 1. It is obvious that L-leucine in the tripeptide glycyl-DL-leucyl-DL-alanine was not available for any one of these two microorganisms and the L-leucine equivalent of the dipeptides DL-leucyl-DL-alanine and DL-alanyl-DL-leucine only partially. In the other DL-leucine peptides the L-leucine was completely available. These results are in agreement with those previously obtained [1], on the medium of Stokes *et al.* Tryptophane in the dipeptide glycyl-L-tryptophane and tyrosine in the dipeptide glycyl-L-tyrosine were also completely utilized. The high blank values in the

Table 2. Lactic acid production of Lactobacillus delbrückii LD5 and Lactobacillus casei in medium supplemented with leucine peptides. Incubation time 72 hours. The medium of Henderson and Snell used in 0.5 times the concentration as given by these authors.

Peptides tested	*Lactobacillus delbrückii* LD5				*Lactobacillus casei*			
	Amounts of free or bound L-leucine per tube in γ							
	0	15	30	60	0	15	30	60
	ml *	ml	ml	ml	ml	ml	ml	ml
L-Leucine	1.5	5.2	7.1	8.9	1.5	5.2	7.1	10.2
DL-Alanyl-DL-leucylglycine	1.5	4.9	7.0	9.1	1.5	5.4	8.2	9.7
Glycyl-DL-leucyl-DL-alanine	1.6	5.5	6.5	9.5	1.9	5.6	7.6	9.7
Glycyl-DL-leucylglycine	1.6	5.6	8.3	10.2	1.8	5.4	8.5	10.3
DL-Leucylglycylglycine	1.4	4.9	7.7	9.9	1.6	5.1	7.9	10.0
DL-Alanyl-DL-leucine	1.5	4.2	7.0	9.7	1.5	5.8	8.0	10.4
DL-Leucyl-DL-alanine	1.8	5.1	6.1	9.8	1.8	5.7	7.6	9.7
Glycyl-DL-leucine	1.6	5.7	7.8	10.8	1.5	4.7	6.9	9.7
DL-Leucylglycine	1.5	5.2	7.0	8.9	1.6	5.0	6.8	10.0
	Complete medium 9.8				Complete medium 10.8			

* Given as ml of 0.05 *N* NaOH to titrate 6 ml of final solution. The values are corrected for the blank titrations.

two tyrosine series are interesting. They seem to demonstrate that on the medium of Henderson and Snell this microorganism can at least partially synthezise tyrosine but better growth is obtained when tyrosine is added to the medium. While Snell [6] previously has reported that this microorganism is less suitable for microbiological determinations of tyrosine, Dunn *et al.*[7] indicate that the amino acid is indispensable for *Streptococcus faecalis* R (8043). The series of leucine peptides assayed with *Lactobacillus delbrückii* LD5 and *Lactobacillus casei* (Table 2) showed that these two microorganisms completely utilized all of the tested peptides. This result is especially interesting when compared with those obtained on the medium of Stokes *et al.*[1]. There is no difference with regard to *Lactobacillus casei* but on the medium of Stokes *et al.* the L-leucine of the tripeptide glycyl-DL-leucyl-glycine was not at all available and the L-leucine of the dipeptide DL-leucyl-glycine only partially available for *Lactobacillus delbrückii* LD5.

Next the leucine peptides were tested on *Leuconostoc mesenteroides* P-60 and *Lactobacillus dextranicum*. The results are given in Table 3. In the series with *Leuconostoc mesenteroides* P-60 there was only two peptides, glycyl-DL-leucyl-glycine and DL-leucyl-glycine where the peptide bound L-leucine had

Table 3. Lactic acid production of Leuconostoc mesenteroides and Lactobacillus dextranicum in medium supplemented with leucine peptides. Incubation time 72 hours. Lactobacillus dextranicum was grown on the medium 0.5 times the concentration as given by Henderson and Snell.

Substances tested	*Leuconostoc mesenteroides*				*Lactobacillus dextranicum*			
	Amounts of free or bound L-leucine per tube in γ							
	0	15	30	60	0	15	30	60
	ml *	ml	ml	ml	ml	ml	ml	ml
L-Leucine	1.1	4.8	6.7	12.2	0	1.7	3.2	5.9
DL-Alanyl-DL-leucylglycine	1.3	3.3	3.8	5.5				
Glycyl-DL-leucyl-DL-alanine	1.3	1.3	1.2	1.1				
Glycyl-DL-leucylglycine	1.3	4.2	6.7	11.4				
DL-Leucylglycylglycine	1.2	3.4	5.5	8.7				
DL-Alanyl-DL-leucine	1.0	3.4	4.7	7.0				
DL-Leucyl-DL-alanine	1.2	2.6	4.2	7.0				
Glycyl-DL-leucine	1.1	3.6	5.8	9.1				
DL-Leucylglycine	1.1	4.5	6.0	11.5	0	0.9	1.0	3.4
	Complete medium 13.0 ml				Complete medium 6.0 ml			

* Given as ml of 0.05 *N* NaOH to titrate 6 ml of final solution. The values are corrected for the blank titrations.

the same activity as the corresponding free amino acid. L-leucine in the tripeptide glycyl-DL-leucyl-DL-alanine was not utilized at all and of the dipeptides the two containing leucine and alanine were clearly less active than the two glycine-leucine peptides. These results are the most convincing of all obtained in the present investigation to indicate that the growth-promoting activity of the tested leucine peptides depends upon the position of the leucine residue with respect to the other amino acid residues, as well as upon the nature of the other amino acid residues. The data obtained with *Lactobacillus dextranicum* are few but none the less of interest. Dunn *et al.*[7] characterize leucine as a dispensable amino acid for this microorganism. However, on the medium of Henderson and Snell it has been found in repeated experiments that leucine has the character of an indispensable amino acid for this microorganism. It is also of interest that DL-leucyl-glycine is so incompletely utilized for growth by this microorganism in contrast to the other lactobacilli so far investigated on the medium of Henderson and Snell.

When the leucine peptides were assayed on *Lactobacillus citrovorum* (8081) and *Lactobacillus citrovorum* (8082) (Table 4) it was found that in both cases

Table 4. Lactic acid production of Lactobacillus citrovorum (8081) and Lactobacillus citrovorum (8082) in medium supplemented with leucine peptides. Incubation time 72 hours. The medium of Henderson and Snell was used in 0.5 times the concentration as given by these authors.

Substances tested	*Lactobacillus citrovorum* (8081)				*Lactobacillus citrovorum* (8082)			
	Amounts of free or bound L-leucine per tube in γ							
	0	15	30	60	0	15	30	60
	ml *	ml	ml	ml	ml	ml	ml	ml
L-Leucine	0.6	3.4	5.2	5.6	0.4	2.8	4.1	6.1
DL-Alanyl-DL-leucylglycine	0.6	4.5	5.9	6.4	0.4	3.5	5.0	6.4
Glycyl-DL-leucyl-DL-alanine	0.5	0.9	1.2	1.2	0.4	2.4	3.5	5.0
Glycyl-DL-leucylglycine	0.6	4.6	5.2	5.2	0.5	2.5	4.4	6.1
DL-Leucylglycylglycine	0.5	5.0	5.5	6.3	0.3	2.9	4.2	6.2
DL-Alanyl-DL-leucine	0.5	2.3	3.8	4.4	0.3	3.3	4.3	5.7
DL-Leucyl-DL-alanine	0.5	1.4	2.4	4.3	0.1	2.2	4.0	5.9
Glycyl-DL-leucine	0.4	4.9	5.5	6.0	0.4	3.3	4.9	6.3
DL-Leucylglycine	0.5	4.9	5.5	6.0	0.5	3.0	4.2	6.0
	Complete medium 5.8 ml				Complete medium 6.8 ml			

* Given as ml of 0.05 *N* NaOH to titrate 6 ml of final solution. The values are corrected for the blank titrations.

the utilization of L-leucine in glycyl-DL-leucyl-DL-alanine was less than that expected if all of the peptide bound L-leucine was available for growth. The leucine equivalents of alanyl-leucine and leucyl-alanine were completely available only for *Lactobacillus citrovorum* (8082). Finally, the series of leucine peptides assayed on *Lactobacillus arabinosus* 17—5 and *Lactobacillus fermenti* 36 (Table 5) showed that the L-leucine content of the tripeptide DL-alanyl-DL-

Table 5. Lactic acid production of Lactobacillus arabinosus 17—5 and Lactobacillus fermenti 36 in the medium of Henderson and Snell supplemented with leucine peptides. Incubation time 72 hours.

Substances tested	*Lactobacillus arabinosus*				*Lactobacillus fermenti*			
	Amounts of free or bound L-leucine per tube in γ							
	0	15	30	60	0	15	30	60
	ml*	ml	ml	ml	ml	ml	ml	ml
L-Leucine	1.8	5.7	8.6	12.7	4.0	7.5	10.8	15.1
DL-Alanyl-DL-leucylglycine	2.3	4.9	6.1	8.0	3.9	3.1	4.5	4.5
Glycyl-DL-leucyl-DL-alanine	2.0	4.8	7.3	11.7	3.7	6.4	8.4	13.0
Glycyl-DL-leucylglycine	1.8	6.2	8.5	13.4				
DL-Leucylglycylglycine	2.2	5.8	10.2	15.9				
DL-Alanyl-DL-leucine	2.0	5.6	9.8	12.5				
DL-Leucyl-DL-alanine	2.0	5.1	7.8	13.1				
Glycyl-DL-leucine	2.0	5.5	8.6	12.0				
DL-Leucylglycine	1.8	5.6	8.5	11.5				
	Complete medium 21.1 ml				Complete medium 20.5 ml			

leucyl-glycine was only partially available for *Lactobacillus arabinosus* 17—5 and was not utilized for growth at all by *Lactobacillus fermenti* 36.

DISCUSSION

The present investigation in agreement with the previous results of Ågren [1] and Krehl and Fruton [2] confirmed the assumption that the capability of some lactic acid bacteria to use leucine of leucine containing peptides supplemented to the basal medium instead of the free amino acid depends upon the position of leucine with respect to the other amino acid residues and upon the nature of the other amino acids. In order to facilitate a comparison semiquantitative

* Given as ml of 0.05 *N* NaOH to titrate 6 ml of final solution. The values are corrected for the blank titrations.

Table 6. Qualitative results of assays of leucine peptides with ten lactic acid bacteria.

++, + and 0 signify the same growth, approximately half the growth respectively no growth effect of peptide bound L-leucine when compared with the effect of the corresponding amounts of free L-leucine.

Micro-organism	Leucine peptides							
	Ala-Leu Gly *	Gly-Leu Ala	Gly-Leu Gly	Leu-Gly Gly	Ala-Leu	Leu-Ala	Gly-Leu	Leu-Gly
(8086)	—	—	—	—	—	—	—	—
(9595)	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +
(7469)	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +
(8082)	+ +	+	+ +	+ +	+ +	+ +	+ +	+ +
(8014)	+	+ +	+ +	+ +	+ +	+ +	+ +	+ +
(8043)	+ +	0	+ +	+ +	+	+	+ +	—
(9790)	+ +	0	+ +	+ +	+	+	+ +	+ +
(8081)	+ +	0	+ +	+ +	+	+	+ +	+ +
(9338)	0	+ +	—	—	—	—	—	+ +
(8042)	+	0	+ +	+	+	+	+	+ +

data of the present investigation are collected in Table 6. Comparing the data in the Table for the ten bacteria it is clear that L-leucine of the tripeptide glycyl-DL-leucyl-DL-alanine is less available for growth than in any of the other tripeptides. DL-alanyl-DL-leucyl-glycine ranked as second in this respect. Leucine of the corresponding dipeptides DL-alanyl DL-leucine and DL-leucyl-DL-alanine was less available than leucine in combination with glycine. The suggestion has been made by Simmonds *et al.*[9] that in the case of many peptides, utilization for growth is preceded by enzymatic hydrolysis to yield essential amino acids. If that is true the combination of leucine and alanine in di- and tripeptides must be considered as rather resistant against the action of lactic acid bacterial peptidases.

Further on, if the suggestion of Simmonds *et al.*[9] is accepted it may be of interest to compare some of the results of the present investigation with the corresponding results previously obtained [1] from this point of view. In both investigations the same strains of bacteria and the same leucine peptides were used. The only difference between the two sets of data was the media, the medium of Henderson and Snell used in the present investigation and that of Stokes *et al.* in the previous work. The use of the two media did not influence the complete utilization of leucine of the eight leucine peptides by *Lactobacillus*

* The abbreviations are those proposed by Brand and Edsall [8].

casei. The results obtained with *Streptococcus faecalis* (9790) were also in agreement. The same leucine peptides stimulated a maximal growth, the same were partially stimulating and in both media there was no growth when leucine was added in the form of glycyl-leucyl-alanine. If an enzymatic hydrolysis of the leucine peptides preceded the utilization, the process seemed not to be influenced by the comparatively small differences in the composition of the two basal media.

Comparing the results obtained on the two media with *Lactobacillus delbrückii* LD5 another picture is obtained. On the medium of Henderson and Snell a complete utilization of leucine peptides was obtained. On the medium of Stokes *et al.* the L-leucine of glycyl-DL-leucyl-glycine was not used at all and the L-leucine of DL-leucyl-glycine only partially. There is no obvious reason why the suggested enzymatic hydrolysis just in this case would be totally or partially blocked on the medium of Stokes *et al.* and completely active on the medium of Henderson and Snell. Here it may only briefly be reminded of the present discussion of the relationship between gene activity and enzyme activity. Small changes in gene activity may be reflected in changes in enzyme activity. The adaptability of bacterial enzymatic processes in certain microorganisms to changes in the hydrogen ion concentration of the surrounding media is well known. On the other hand the medium of Henderson and Snell contains considerably higher concentrations of mangan and magnesium ions, both known as activators of bacterial peptidases. The variability of results obtained when analysing the same synthetic peptides by the same microorganism on two slightly different media may also occur when studying a biological active peptide on different media [5].

The present investigation also demonstrated the difficulties in characterizing an amino acid as dispensable or indispensable. According to Dunn *et al.* leucine is a dispensable amino acid for *Lactobacillus dextranicum* on the medium specified by these authors. In the present investigation it was shown that on the medium of Henderson and Snell leucine is an indispensable amino acid for this microorganism. According to Dunn *et al.* tyrosine is an indispensable amino acid for *Streptococcus faecalis* R (8043) while on the medium of Henderson and Snell the microorganism is able to grow when this amino acid is not present in the medium. Finally, it may again be stressed that the type of analysis illustrated in Table 6 may be useful for the identification of the order in which amino acids are linked together. The peptides analyzed in this work did not show strepogenin activity.

SUMMARY

Analysis of the utilization of various peptides especially leucine peptides by ten lactic acid bacteria have shown that the growth-promoting activity of such peptides depends on the position of leucine in the peptide and on the nature of the surrounding amino acids. This type of analysis may also be useful for the establishment of the order in which the amino acids in peptides are linked together. It is also demonstrated that the utilization of a peptide bound essential amino acid may be changed by cultivation on slightly different media. Changes in dispensability and indispensability of amino acids for lactobacilli cultivated on different media were also observed.

Parts of the expenses for this investigation were defrayed by a grant from *Statens naturvetenskapliga forskningsråd.*

REFERENCES

1. Ågren, G. *Acta Physiol. Scand.* **13** (1947) 347.
2. Krehl, W. E., and Fruton, J. S. *J. Biol. Chem.* **173** (1948) 179.
3. Stokes, J. L., Gunness, M., Dwyer, I. M., and Caswell, M. C. *J. Biol. Chem.* **160** (1945) 35.
4. Henderson, L. M., and Snell, E. E. *J. Biol. Chem.* **172** (1948) 15.
5. Ågren, G. In press.
6. Snell, E. E. *Ann. N. Y. Acad. Sci.* **47** (1946).
7. Dunn, M. S., Shankman, S., Camien, M. N., and Block, H. *J. Biol. Chem.* **168** (1947) 1.
8. Brand, E., and Edsall, J. T. *Ann. Rev. Biochem.* **16** (1947) 223.
9. Simmonds, S., Tatum, E. L., and Fruton, J. S. *J. Biol. Chem.* **170** (1947) 483.

Received August 30, 1948.