IDENTIFICATION OF THREO-3-HYDROXYGLUTAMIC ACID IN THE CELL WALL OF MICROBACTERIUM LACTICUM

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The murein (peptidoglycan) of Microbacterium lacticum shows an unusual amino acid composition (Schleifer et al. 1967). Besides the usual amino sugars it contains the amino acids lysine, glycine, D-alanine, small amounts of glutamic acid and an hitherto unknown amino acid. The latter behaves chromatographically very similar to aspartic acid. In fact Keddie et al. (1966) claim that they have found aspartic acid in the cell wall of Mb. lacticum.

This paper describes the identification of the unknown substance as threo-3-hydroxyglutamic acid. So far this compound has only been found in human brains and in brains of cows (Ohara et al. 1962).

Experimental and results:

Mb. lacticum 6 isolated from pasteurized milk, and Mb. lacticum ATCC 8180 were grown in a yeast extract dextrose broth (Schleifer et al. 1967) at 30° C under aerobic conditions (shaker) and harvested in the stationary phase. Cell walls were prepared by the usual technique (Cummins and Harris 1956). The cells were disintegrated with glass beads and the cell walls harvested by centrifugation. Further purification was achieved by incubation with trypsin for 24 hrs and extraction with 10 % trichloroacetic acid (3 days at 4° C).

Paper chromatography was carried out on SS 2043b in the following solvent systems:

1.	Isopropanol/acetic acid/water	75:10:15
2.	α-Picoline/conc. NH_4OH/water	70:2:28
3.	88 % Phenol/acetic acid/EDTA/water	168:32:0,2:2
4.	n-Butanol/acetic acid/water (upper phase)	4:1:5
5.	Methanol/pyridine/formic acid/water	80:10:1:19

Paper electrophoresis was performed under the following conditions: sandwich type electrophoresis, 400 V (10 V/cm); 7 hrs; electrolyte: formic acid/acetic acid/water 5:15:80, pH 1,9; filter paper SS 2043b.

The total hydrolysate (4 N HCl, 100° C, 16 hrs) of TCA-extracted cell walls contains a ninhydrin-positive compound which moves in solvent systems 1 and 2 like aspartic acid, but the spot shows a brownish color after spraying with ninhydrin reagent (95 ml water-saturated n-butanol, 5 ml acetic acid, 0,5 g ninhydrin) as compared to the steel-blue color of aspartic acid.

For further identification the compound was isolated from the total hydrolysate by preparative paper chromatography in solvent systems 1 and 2. Samples of the pure compound and of threonine and serine were subjected to paper chromatography and sprayed with alkaline silver nitrate (Trevelyan et al. 1950). The compound shows a positive reaction like hydroxyamino acids. By spraying with alkaline periodate the release of ammonia could be demonstrated with Nessler's reagent. This indicates the presence of a hydroxyl group and an amino group at adjacent carbon atoms (Nicolet and Shinn, 1939). On hydrolysis of the compound with saturated $Ba(OH)_2$ some glycine was formed. This reaction is typical for ß-hydroxyamino acids (Wieland and Wirth, 1949). The compound was stable when

treated 18 hrs with 6 N HCl at 120° C. During electrophoresis, the unknown substance behaved like a dicarboxylic amino acid.

On the basis of these results we assumed that the unknown substance is a ß-hydroxydicarboxylic amino acid. ß-hydroxyaspartic acid could be excluded since both isomers of this compound show 2 peaks in column chromatography (Hamilton, 1963), while our compound forms one peak only.

Therefore, we compared the unknown compound with an authentic sample of threo- as well as erythro-3-hydroxyglutamic acid, which were kindly supplied by Merck, Sharp & Dohme, Rahway, New Jersey. Tab. 1 shows the R_{Ala}-values and the distance of migration in electrophoresis of the two isomers and of glutamic and aspartic acid. The unknown substance behaved in all systems mentioned in Tab. 1 like threo-3-hydroxyglutamic acid.

In addition the cochromatography of the unknown substance on the column (amino acid analyzer, Bender & Hobein München) showed complete coincidence with threo-3-hydroxyglutamic acid. The elution sequence of aspartic acid, threonine and the two hydroxy derivatives is shown in Fig. 1.

To demonstrate that threo-3-hydroxyglutamic acid is in fact a constituent of the murein, some peptides of the partial hydrolysate (4 N HCl; 1 hr; 100° C) of the murein were analyzed. Glycyl-threo-3-hydroxyglutamic acid could be identified which corresponds to glycyl-glutamic acid found in the partial hydrolysate of the cell wall precursor as described previously (Schleifer et al. 1967). In addition, we also found N^2-threo-3-hydroxy-glutamyl-lysin. This indicates that in the murein some of the glutamic acid is replaced by its hydroxy derivative. The hydroxy-

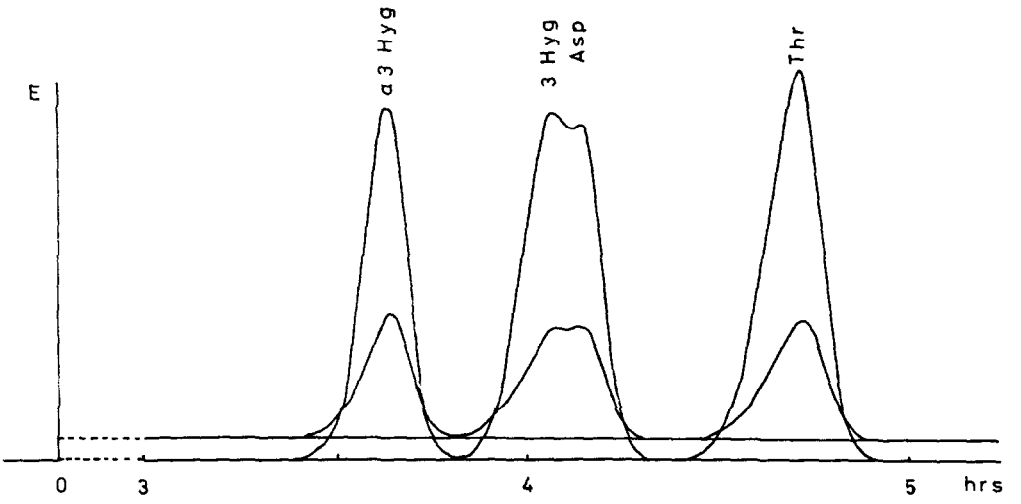

Fig. 1: Column chromatographic separation of threo-3-hydroxyglutamic acid (synonymous to allo-3-hydroxyglutamic acid; a 3 Hyg), erythro-3-hydroxyglutamic acid (3 Hyg), aspartic acid and threonine (Amino acid analyzer; Bender and Hobein München; citrate buffer pH 3,14)

Tab. 1: Migration of various amino acids in paper chromatography (R_{Ala}-values) and in paper electrophoresis (m_{Ala}-values)

Amino acids	solvent systems 1	2	3	4	5	Electrophoresis
Glutamic acid	0,78	0,38	0,59	0,88	0,84	0,65
Erythro-3-hydroxyglutamic acid	0,56	0,30	0,30	0,73	0,75	0,57
Threo-3-hydroxyglutamic acid	0,49	0,29	0,31	0,75	0,67	0,53
Aspartic acid	0,54	0,32	0,32	0,74	0,59	0,60
Unknown compound	0,49	0,29	0,31	0,75	0,67	0,53

lation seems to occur once the murein is formed since the precursor, accumulating after inhibition by D-cycloserine, only contains glutamic acid. The ratio of glutamic acid to threo-3-hydroxyglutamic acid in the murein depends very much on the oxygen supply during growth (Schleifer and Kandler, paper in preparation).

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