D-Glutamic acid and D-alanine as constituents of spores of *Bacillus megatherium*

Water-soluble, non-dialysable components ("spore peptide") were isolated from extracts of disintegrated resting spores and exudates of germinating spores of *Bacillus* species[1]. After acid hydrolysis, they contained $\alpha\varepsilon$-diaminopimelic acid, glutamic acid, alanine and acetyl derivatives of hexosamine and a substituted hexosamine[2,3]. There is some evidence that "spore peptide" is associated with the coat of the resting spore[4], which is interesting in view of the presence of the same amino acids and amino sugars in acid hydrolysates of the isolated cell walls of a number of vegetative, gram-positive organisms[5,6]. The compositions of the isolated cell walls of *Lactobacillus casei* and *Streptococcus faecalis* have been investigated recently by SNELL, RADIN AND IKAWA[7] and IKAWA AND SNELL[8] who found that considerable amounts of the total glutamic acid and alanine present in acid hydrolysates were the D-isomers. PARK[9] reported the presence of both D-alanine and D-glutamic acid together with amino sugars in a uridine-5'-phosphate component produced by penicillin-treated *Staphylococcus aureus*. In view of these findings it was pertinent to look for the presence of D-alanine and D-glutamic acid in "spore peptide". In this investigation, *B. megatherium* "spore exudate peptide" was used; this, according to available physical criteria, is homogenous with a molecular weight of 15,300[10].

The usual method of estimating D-glutamic acid has been the indirect one of subtracting the amount of the L-isomer, as determined with decarboxylase, from the total amount of glutamic acid. The recent finding by MIZUSHIMA *et al.*[11] of a D-glutamic acid oxidase in *Aspergillus ustus* makes possible for the first time a convenient and accurate method for the direct determination of D-glutamic acid. The results reported here demonstrate the usefulness of this method, which was used in conjunction with the decarboxylase method to obtain positive analyses of both isomers.

B. megatherium "spore peptide" (200 mg) was hydrolysed by heating in a sealed tube with 6 *N* HCl (3 ml) for 20 h and the HCl was removed by distillation *in vacuo*. Glutamic acid and alanine were isolated from the hydrolysate by ion exchange chromatography. In one procedure the hydrolysate was passed through a column of Amberlite IR-120(H) and the amino acids were eluted with pyridine (10% v/v). The pyridine was removed from the amino acids fraction by distillation to dryness *in vacuo* and an aqueous solution of the residue was passed through a column of IR-4B(OH). Glutamic acid was eluted from the column with 1 *N* HCl. In another procedure the amino acids were separated by elution from a column of Dowex-50(H) with 0.3 *N* HCl. Glutamic acid was partially freed from HCl by evaporation *in vacuo* but was not purified further. Alanine from the Dowex-50 column was freed from HCl by means of IR-4B(OH) and crystallized from ethanol-water.

Total glutamic acid was determined by paper chromatography[12]. D-Glutamic acid was determined manometrically by means of D-glutamic acid oxidase from *Asp. ustus*[11]. Fresh mycelium of *Asp. ustus* strain *f* was ground with alumina powder in 0.05 *M* phosphate, pH 8.0, and treated as described by these authors. The volume of the final extract was reduced about 50% by lyophilization. The preparation had little or no endogenous activity and remained highly active towards D-glutamic acid for 4 months when stored at —20° C. The preparation was completely inactive with L-glutamic acid, DL-alanine and all isomers of $\alpha\varepsilon$-diaminopimelic acid. The preparation (0.2 or 0.3 ml) was added to the side arm of a Warburg vessel and the sample at pH 8.0 was placed in the main compartment. The final volume was made up to 2.3 ml with 0.05 *M* phosphate buffer, pH 8.0.

L-Glutamic acid was determined manometrically with decarboxylase[13] but a lyophilized sonic extract of *Escherichia coli* was used instead of dried cells.

D-Alanine was determined manometrically with D-amino acid oxidase from sheep kidneys[14]. No specific estimation of the L-isomer was made but the alanine remaining after incubation with D-amino acid oxidase under conditions allowing the complete oxidation of the same amount of D-alanine was estimated by paper chromatography with collidine-lutidine-H_2O as solvent. Spots corresponding in position to alanine were located with ultraviolet light and cut out and their amino acid content was determined.

Quantitative assays of glutamic acid and alanine in hydrolysates are given in Table I. 99% of the glutamic acid and 24% of the alanine were the D-isomers. IKAWA AND SNELL[8] reported that hydrolysis of casein in 3 *N* HCl in the presence of an equal weight of starch racemized 12–20% of the L-glutamic acid. *B. megatherium* "spore peptide" is rich in amino sugars but contains only a small amount of other carbohydrate[1]. However, the possibility of racemization occurring during acid hydrolysis was checked by heating mixtures of amino sugars, amino acids and glucose in acid, using approximately the concentrations found in hydrolysates of "spore peptide" (Table I). Under these conditions little or no racemization occurred.

An attempt was made to release glutamic acid from the peptide by an enzyme preparation from *Bacillus subtilis* which hydrolyses γ-D-glutamyl peptides[15]. When incubation mixtures of

TABLE I

ANALYSIS OF GLUTAMIC ACID AND ALANINE FROM ACID HYDROLYSATES OF "SPORE PEPTIDE" AND SIMULANT MIXTURES

Sample	*Amino acid assayed*	*Total*	D-*Isomer*		L-*Isomer*		D- + L-*Isomers*
		μmole/ml	*μmole/ml*	*% of total*	*μmole/ml*	*% of total*	*% of total*
Peptide hydrolysate I	Glutamic acid	4.20	4.17	99.2	0.12	2.9	102.1
	Alanine	14.90	3.61	24.2			
	Alanine	7.58			5.73*	75.6	99.8
Peptide hydrolysate II	Glutamic acid	4.53	4.51	99.6	0.12	2.6	102.2
	Alanine	33.40	7.95	23.8			
	Alanine	8.43			6.69*	79.4	103.2
Mixture A	Glutamic acid	4.80	0	0			
Mixture B	Glutamic acid	13.60			0.08	0.6	
Mixture C	Alanine	8.43			8.87*	105.2	
D-Glutamic acid	Glutamic acid	10.0	9.95	99.5			
L-Glutamic acid	Glutamic acid	5.0			4.99	99.8	

Peptide hydrolysate I: Glutamic acid and alanine isolated on IR-120(H) and IR-4B(OH).
Peptide hydrolysate II: Glutamic acid and alanine isolated on Dowex-50. Alanine crystallized from ethanol-water.
Mixture A: DL-alanine, 15 mg; glucosamine HCl, 50 mg; $\alpha\varepsilon$-diaminopimelic acid, 10 mg; L-glutamic acid, 10 mg; glucose, 1.5 mg; acetic acid, 15 mg. Mixture heated with 4 ml of 6 *N* HCl for 16 h at 105°.
Mixture B: Same as A except that D-glutamic acid was used.
Mixture C: L-alanine, 2.1 mg; glucosamine HCl, 7 mg; $\alpha\varepsilon$-diaminopimelic acid, 1.4 mg; D-glutamic acid, 1.5 mg; glucose, 0.6 mg. Treated with HCl as for A and B.

* Alanine remaining after treating with D-amino acid oxidase. When D-alanine was treated under the same conditions, it was completely oxidized.

this enzyme and "spore peptide" were analysed, interesting results were obtained. Paper chromatograms developed with phenol-water showed a spot corresponding in position to glutamic acid but tests with D-glutamic acid oxidase failed to confirm the presence of D-glutamic acid in the incubation mixtures. When the spot was eluted from the paper, further investigation showed that it was not free glutamic acid but a peptide or mixture of peptides containing glutamic acid, alanine, $\alpha\varepsilon$-diaminopimelic acid and amino sugars. At least 4 other spots were obtained on paper chromatograms of these enzymic hydrolysates and preliminary investigations indicate that the peptidase will be a useful tool for the release of small molecular weight peptides. The study of these will help in the elucidation of the structure of "spore peptide".

We are grateful to Dr. K. SAKAGUCHI for supplying us with a culture of *Aspergillus ustus* strain *f*.

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Received January 4th, 1957

* During a year's working visit from Fort Detrick, Frederick, Maryland, U.S.A.