A NEW SPECIES (*FLAVOBACTERIUM POLYGLUTAMICUM*) WHICH HYDROLYZES THE γ-L-GLUTAMYL BOND IN POLYPEPTIDES

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It has been shown (Kovács and Bruckner, 1952; Bruckner *et al.*, 1953*a*, 1953*b*, 1953*c*) that certain bacteria produce glutamyl polypeptides in which the γ-glutamyl bond predominates; that produced by *Bacillus anthracis* (Ivánovics and Erdös, 1937; Ivánovics and Bruckner, 1937; Hanby and Rydon, 1946) and by *Bacillus mesentericus* (Bruckner and Ivánovics, 1937) contains the D-isomer alone, whereas that formed by *Bacillus subtilis* (Bovarnick, 1942) contains both D- and L-isomers in varying quantities (Thorne *et al.*, 1954). Confirmatory evidence for the presence of the γ-glutamyl bond has been provided recently by work with synthetic γ-poly-L- (Waley, 1955; Bruckner *et al.*, 1955*a*) and γ-poly-D-glutamic acid (Bruckner *et al.*, 1955*b*). Other natural products in which γ-glutamyl residues occur include pteroylglutamic acid conjugates and *p*-aminobenzoylpolyglutamic acid. These substances are important growth factors and are split by conjugases (Stokstad, 1954). It was thought that an organism that can grow on the bacterial polypeptide and can specifically split the γ-glutamyl linkages might serve as the source of an enzyme to demonstrate the existence of γ-glutamyl bonds in proteins.[2] Indeed, it has been reported (Haurowitz and Bursa, 1949) that γ-linked glutamic acid occurs in proteins, but this has not been conclusively demonstrated since there is as yet no reliable method for distinguishing between α- and γ- bonds in protein; an improved chemical method was recently described by Haurowitz and Horowitz (1956).

[1] Part of a thesis submitted by P. Margalith to the Hebrew University, Jerusalem, in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

[2] Not long after this work was began, Thorne *et at.* (1954) found, in culture-filtrates of *Bacillus subtilis*, an enzyme that hydrolyzes the native glutamyl polypeptides; Kream *et al.* (1954) found a similar enzyme in human red blood cells and in various tissues. However, the specificity of these enzymes to substrates other than polyglutamic acid was not reported.

In seeking such an organism, a new species of Flavobacterium was isolated; grown on γ-poly-DL-glutamic acid, it produces an enzyme which specifically splits the γ-glutamyl linkages in the L-polypeptide. The bacterium is described and the partial purification of the enzyme and its properties are reported.

MATERIALS AND METHODS

Isolation and description of Flavobacterium polyglutamicum n. sp. Enrichment cultures, inoculated with forest soil, were prepared in a medium containing K_2HPO_4, 0.1 g; $MgSO_4$ ($7H_2O$), 0.05 g; $CaCl_2(2H_2O)$, 0.01 g in 100 ml distilled water, pH 7.4. γ-Poly-DL-glutamic acid was added aseptically, from a 2.5 per cent sterile solution, to a final concentration of 0.2 per cent. The solution was prepared as follows: the glutamyl polypeptide was well suspended in distilled water and dissolved by slow addition of a few drops of a 10 per cent sodium hydroxide solution, the pH being kept under 9.0. When all the substance was dissolved, the pH was adjusted to 7.0, and the solution was autoclaved for 20 min at 120 C. Paper chromatography showed that no free glutamic acid was formed after such a treatment.

The cultures were incubated at 28 C, and abundant growth appeared within 48 hr. Three successive transfers were made in the same medium at 48-hr intervals, and finally pure cultures were obtained by streaking on the same medium supplemented with 3.0 per cent agar.

Several different bacteria capable of utilizing polyglutamic acid as a sole source of nitrogen and carbon were isolated. These organisms usually produced mucinous substances whether grown in solid or liquid media; this prevented the packing of the cells even at high-speed centrifugation (30 min at 23,000 × G). The organism described herewith was the only one which could be collected in the Sharples supercentrifuge.

Standard methods of identification were em-

ployed for determination of the taxonomic position of the organism.

The organism has the following characteristics: rods, 0.15 to 0.45 μ by 1.5 to 2.0 μ, occurring singly and in pairs; nonmotile, nonspore-forming, gram-negative; colonies on polyglutamic acid agar (after 48 hr), punctiform, smooth, entire, convex, opalescent, slightly yellowish; on nutrient agar (after 3 days), colonies somewhat larger than above; on nutrient agar with 0.5 per cent glucose, the colonies are lemon yellow, the color becoming more intense as the culture ages (the pigment does not diffuse into the medium); on nutrient agar slant; moderate, slightly echinulate, raised, smooth, opalescent, butyrous, grayish turning yellowish; in the presence of 0.5 per cent glucose, an intense lemon-yellow color is formed; on gelatin stab, scanty surface growth, no liquefaction; in nutrient broth, turbid, thin pellicle, granular sediment. Litmus milk turns strongly alkaline; no peptonization. Nutritional requirements: no growth with acetate, succinate, citrate, or glucose (0.4 per cent) as the source of carbon, and ammonium nitrate (0.2 per cent) as the source of nitrogen, in the synthetic medium used for the enrichment cultures. Moderate growth in pyruvate and abundant growth in lactate. Forms acid without gas from glycerol, mannitol, and salicin (tested in a synthetic medium: $NH_4H_2PO_4$, 0.1 g; KCl, 0.02 g; $MgSO_4(7H_2O)$, 0.02 g in 100 ml distilled water; supplemented with 0.2 per cent yeast extract (Difco) and Andrade's indicator). No acid or gas from xylose, glucose, galactose, mannose, or sucrose. Starch not hydrolyzed. Indole not formed. Nitrites moderately produced from nitrates; no gas is formed. Hydrogen sulfide (in Kligler's iron agar slant) not formed. Acetylmethylcarbinol not produced. Urease negative. Ammonia positive. Catalase positive. Strict aerobe. Optimal temperature, 28 C; scanty growth at 37 C. Pigment insoluble in ether, acetone, or benzene; soluble in glacial acetic acid; a blue color given with concentrated sulfuric acid, thus indicating a carotenoid.

The characteristics of the organism resemble those of the genus *Flavobacterium* as recently redefined by Weeks, (1955). Since the organism could not be identified with any previously described Flavobacterium in the current edition of *Bergey's Manual* (Breed *et al.*, 1948), it is proposed that this organism be named *Flavobacterium polyglutamicum* n. sp. The specific epithet indicates the ability of the organism to utilize γ-poly-DL-glutamic acid.

Compounds. The various peptides and polypeptides used in this study were obtained from different sources as indicated in table 3.

Preparation of γ-poly-DL-glutamic acid. The polypeptide was obtained from 4-day-old cultures of *B. subtilis* ATCC strain 9945, grown in 3-L Fernbach flasks containing 500 ml of Sauton's medium (Bovarnick, 1942) and incubated at 34 C. The polypeptide was isolated according to Bovarnick's method (1942) with the following modifications: the gummy copper precipitate of the polypeptide was dissolved by grinding in a small volume of 1.5 N HCl, and the insoluble residue was immediately removed by centrifugation. The clear supernatant fluid was kept at 2 C; within 24 to 48 hr a white flaky precipitate appeared. The fluid was decanted and the precipitate collected by centrifugation. It was washed twice with 1.5 N HCl, twice with distilled water, and then dialyzed, with stirring, against a large volume of distilled water for 36 hr at room temperature. It was finally collected by centrifugation and dried over P_2O_5 *in vacuo*. The yield was 1.3 to 1.5 g of polypeptide per 1 L culture medium. Two-dimensional paper chromatography of acid hydrolyzates of this material in phenol-water (7:3 w/v) and propanol-water (7:3 v/v) showed only glutamic acid to be present; hydrolysis was performed in 6 N HCl at 120 C for 8 hr.

The preparation contained 6 per cent moisture and 10.1 per cent nitrogen (calculated N, 10.8 per cent). Upon acid hydrolysis 90 per cent glutamic acid was released. All quantitative data for glutamic acid given below are corrected for these values.

The optical rotation of the acid hydrolyzate, C 5.4 g per 100 ml of 6 N HCl, ranged between $[\alpha]_D^{20} = +1°$ and $+4°$ for various preparations. All the experiments reported below were carried out with a sample having the optical rotation of $[\alpha]_D^{20} = +1.48°$, showing that it contained about 52 per cent L-isomer. Additional determinations of the amount of L-glutamic acid in the polypeptide hydrolyzates were carried out with glutamic acid decarboxylase; the amount varied between 46 and 52 per cent.

The molecular weight of various preparations, determined according to Sanger's fluorodinitrobenzene method for amino end-group analysis, was found to vary between 10,500 and 12,000.

The procedure described by Leube *et al.* (1954) was used. DNP-Polyglutamic acid was prepared according to Waley (1955), and DNP-L-glutamic acid, according to Levy (1954).

Cultural methods. Stock cultures of *F. polyglutamicum* were maintained on nutrient agar (Difco) slants. Mass cultures were grown in lots of 6-L Erlenmeyer flasks containing 3 L of 0.5 per cent yeast extract (Difco) in distilled water. The inoculum consisted of a 24-hr-old culture grown on nutrient agar in a Roux bottle. The cultures were aerated vigorously for 16 hr at 28 C. Cells were harvested in a Sharples supercentrifuge; they were washed by running 1 L of chilled distilled water through the centrifuge. If not immediately extracted, they were stored at −20 C until needed. The yield was approximately 5 g of wet bacteria per L of culture.

Preparation of cell free extracts. Extracts were obtained by subjecting suspensions of 8 g wet cells in 30 ml 0.9 per cent KCl to sonic vibration (Submarine Signal 9 kc sonic oscillator) for 60 min at 5 C. Cell debris was removed by centrifugation in a Servall type SS-1 centrifuge at 2 C and 23,000 × G for 30 min. The clear, yellowish, slightly viscous supernatant, containing between 6 to 10 mg protein per ml, constituted the crude enzyme preparation; it maintained its activity for several months when stored at −20 C.

Analytical methods. Total glutamic acid was determined by the colorimetric ninhydrin method of Moore and Stein (1948) except that a 1 per cent ninhydrin solution was employed, and the resulting reaction mixture was heated in a boiling water bath for 5 min. Readings were taken on the Klett-Summerson photoelectric colorimeter with a No. 56 filter (maximum transmission at 560 mμ), using L-glutamic acid as the standard. L-Glutamic acid was estimated manometrically with glutamic acid decarboxylase (specific for the L-isomer) obtained from *Escherichia coli* ATCC strain 9637, as described by Umbreit and Gunsalus (1945). The incubation mixture was heated for 5 min in boiling water, cooled, and adjusted to pH 4.5 with a 15 per cent H_2SO_4 solution. The precipitate was removed by centrifugation, and the supernatant fluid was used for the determination. D-Glutamic acid was determined by subtracting the amount of the L-isomer from the total amount of glutamic acid present.

Protein in the crude and purified enzyme was determined turbidimetrically by the trichloracetic acid method of Stadtman *et al.* (1951). Armour's crystalline bovine plasma albumin was used as the standard.

Paper chromatography. Samples of the incubation mixture were placed on strips of Whatman no. 1 filter paper. Unidimensional descending chromatograms were run at room temperature for 16 hr. Depending on the substance, either of two solvent systems was employed: (1) *n*-butanol-acetic acid-water (4:1:1 v/v); (2) phenol, saturated with water and aged for at least one week before use. Amino acid spots were located on the chromatograms by spraying with 0.25 per cent ninhydrin in *n*-butanol containing a few drops of collidine. *p*-Aminobenzoylglutamic acids were revealed by spraying with Ehrlich's reagent containing 1 g *p*-dimethylaminobenzaldehyde in 95 ml of ethanol (95 per cent) and 20 ml of concentrated hydrochloric acid.

Enzyme assay. For the assays, small test tubes were used, each containing M/30 Veronal buffer, pH 8.5, and various quantities of substrate and enzyme, as reported for each experiment. Controls, consisting of enzyme without substrate and substrate without enzyme, were included in all experiments. Their values were subtracted from those of the complete system; only corrected values are reported. The system was incubated at 37 C. The reaction was stopped by adding 0.1 ml portions of each of the samples to 1.0 ml of the ninhydrin reagent and immediately heating the resulting mixture in a boiling water bath for 5 min.

Unit. A unit of enzyme activity is defined as that amount of enzyme which will liberate 0.1 μmoles glutamic acid from 0.6 mg polyglutamic acid in 15 min under the conditions of the standard assay.

Specific activity. Specific activity is defined as the number of activity units per mg of protein.

RESULTS

Purification of enzyme. All operations were carried out in a cold room kept at 2 to 4 C, and all centrifugations were conducted at 23,000 × G for 30 min. The purification procedure was as follows: to 60 ml of the sonic extract, 3 ml of a 1 M $MnCl_2$ solution was slowly added with constant stirring, and stirring was continued for 30 min. The resulting precipitate was removed by centrifugation. The clear supernatant fluid was adjusted to pH 7.0 with a 30 per cent solu-

tion of K_2CO_3; 40 ml of ammonium sulfate solution, saturated at 2 C and neutralized with K_2CO_3 to pH 7.0, was very slowly added with stirring, to give a final saturation of 0.40. The stirring was continued for 2 hr, and the precipitated material was then collected by centrifugation. This precipitate was dissolved in 5 ml M/30 Veronal buffer, pH 8.5, and dialyzed for 20 hr with stirring, against several changes of the same buffer. A small amount of precipitate resulted, and was removed by centrifugation, leaving a clear, viscous, yellow solution. Table 1 summarizes the results of a typical fractionation. This method of purification permits the recovery of 60 to 80 per cent of the enzyme present in crude extracts, with an 8- to 10-fold increase in purity. All the experiments reported below were carried out with a similarly purified preparation.

TABLE 1

*Summary of data on enzyme purification**

Fraction	Volume	Protein	Enzyme Units	Specific Activity	Recovery of Original Units
	ml	*mg*			%
Crude extract...	60	360.0	504	1.4	100
Supernatant, $MnCl_2$.....	59	165.2	429	2.6	85
$(NH_4)_2SO_4$ ppt. 0.40 sat.....	5	31.0	385	12.4	76

* Assay system contained 0.6 mg γ-poly-DL-glutamic acid, enzyme preparation, and M/30 Veronal buffer, pH 8.5, in a total volume of 1.0 ml. Incubation for 15 min at 37 C.

TABLE 2

*Amount of glutamic acid released from γ-poly-DL-glutamic acid determined with ninhydrin and glutamic acid decarboxylase at various times of incubation.**

Method	Time of incubation (min)			
	30	60	120	240
	μmoles	*μmoles*	*μmoles*	*μmoles*
Ninhydrin	2.7	6.2	7.9	10.7
Glutamic acid decarboxylase	2.9	5.8	8.5	11.4

* Reaction mixture contained 18 mg polyglutamic acid, 3 mg enzyme protein, M/30 Veronal buffer, pH 8.5, in a total volume of 3.0 ml. Incubated at 37 C for the times noted.

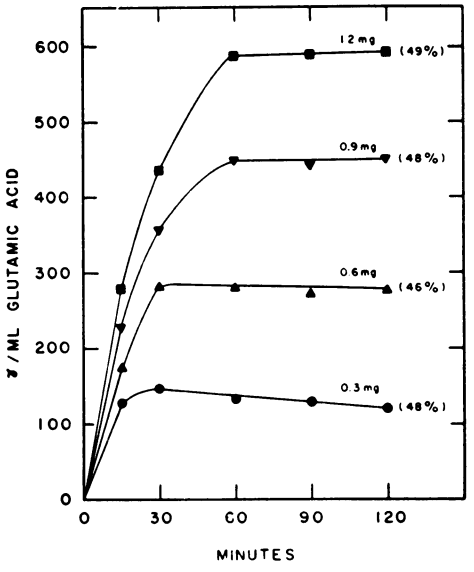

Figure 1. Rate of hydrolysis of γ-poly-DL-glutamic acid. Reaction mixture contained 1 mg protein, M/30 Veronal buffer, pH 8.5, and different amounts of polyglutamic acid as indicated above each line, in a total volume of 1.0 ml. Incubation at 37 C for the times noted. The ninhydrin color procedure was used in following the hydrolysis, and the glutamic acid decarboxylase method was used to determine the amount of L-glutamic acid released at the end of the incubation period. The figures in parentheses represent the amount of L-glutamic acid liberated, calculated as per cent of the total glutamic acid present in the polypeptide.

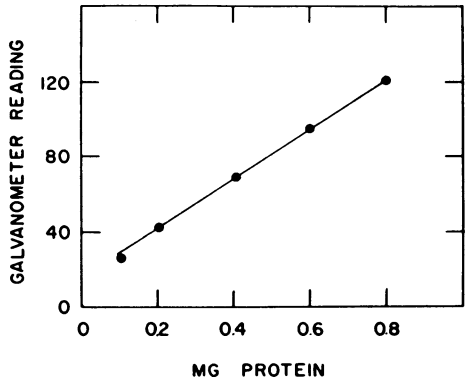

Figure 2. Effect of enzyme concentration on the hydrolysis of polyglutamic acid. Reaction mixture contained 0.6 mg polyglutamic acid, M/30 Veronal buffer, pH 8.5, and the indicated amounts of enzyme in a total volume of 1.0 ml. Incubation for 10 min at 37 C. Hydrolysis determined by the ninhydrin color procedure.

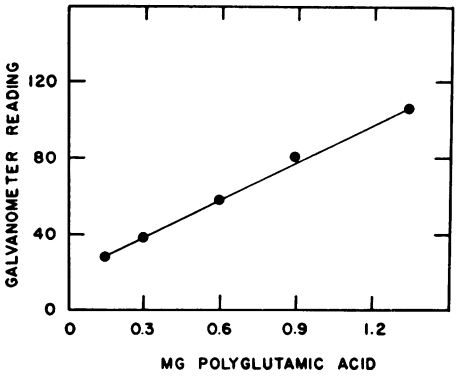

Figure 3. Effect of substrate concentration on the hydrolysis of polyglutamic acid. Reaction mixture contained 0.5 mg enzyme protein, M/30 Veronal buffer, pH 8.5, and the indicated amounts of polyglutamic acid in a total volume of 1.0 ml. Incubation for 10 min at 37 C. Hydrolysis determined by the ninhydrin color procedure.

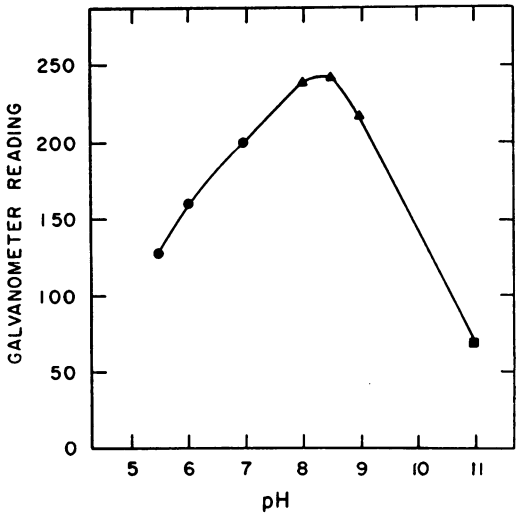

Figure 4. Effect of pH on the hydrolysis of polyglutamic acid. Reaction mixture contained 0.6 mg polyglutamic acid, 1 mg enzyme protein, and M/30 buffer in a total volume of 1.0 ml. Incubation for 60 min at 37 C. The pH was measured at the end of the reaction with a glass electrode. The buffers used were: (●) phosphate, (▲) Veronal, and (■) bicarbonate-carbonate. Hydrolysis determined by the ninhydrin color procedure.

Properties of the enzyme. The enzyme hydrolyzing polyglutamic acid is constitutive since it occurs in cells grown on synthetic medium containing lactate and ammonium nitrate, but no polyglutamate. The purified enzyme becomes partially denatured when kept at −20 C. It loses most of its activity after a few days, when stored at 2 C, and a yellow precipitate is formed. The enzyme is completely inactivated by heating at 60 C for 3 min (tested with 0.5 mg enzyme protein per ml).

Identification of reaction product. Polyglutamic acid was incubated with the enzyme, and aliquots of the incubation mixture were removed after 5, 10, 20, 30 min, and 1, 2, 4, and 6 hr, by which time the hydrolysis was complete. Paper chromatograms showed in each case only a single ninhydrin-positive spot, identical with L-glutamic acid (R_f 0.32 in butanol-acetic acid-water, and 0.28 in phenol-water, run up to 3 days). In addition, ninhydrin determinations and manometric estimations with glutamic acid decarboxylase carried out simultaneously to verify the release of L-glutamic acid, showed a good agreement between the two methods (table 2).

Rate of hydrolysis. Figure 1 shows the rate of release of L-glutamic acid at different concentrations of polyglutamic acid. It may be seen that the rate of the reaction is linear for only a short period and thereafter falls off rapidly. The amount of L-glutamic acid formed corresponded

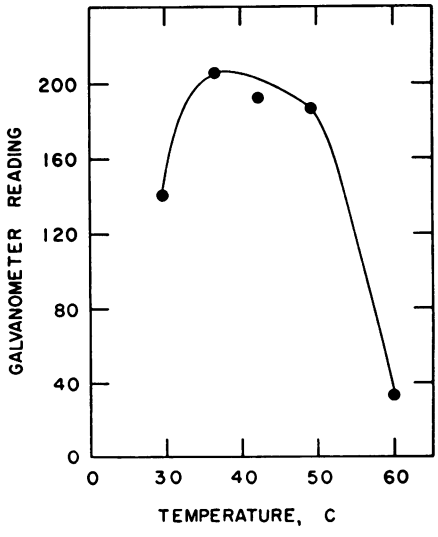

Figure 5. Effect of temperature on the hydrolysis of polyglutamic acid. Conditions as described in figure 4 except that M/30 Veronal buffer, pH 8.5, was used, and temperatures were as indicated.

TABLE 3

Action of the bacterial enzyme on various types of substrates

Compound	Source	Breakdown*	Remarks
Peptides†			
Glycyl-glycine	Mann Research Labs., Inc.	++	
Glycyl-L-phenylalanine	Mann Research Labs., Inc.	++	
L-Alanyl-glycine	Mann Research Labs., Inc.	++	
Glycyl-glycyl-glycine	Mann Research Labs., Inc.	++	
α-L-Glutamyl-L-glutamic acid	Dr. H. Sachs	++	
γ-L-Glutamyl-L-glutamic acid	Dr. H. Sachs	++	
γ-L-Glutamyl-γ-L-glutamyl-L-glutamic acid	Dr. H. Sachs	++	
Glutathione (γ-L-glutamyl-L-cysteinyl-glycine)	Nutritional Biochemicals Corp.	−	
γ-L-Glutamyl-cysteine	Dr. F. J. R. Hird	−	
		per cent	
Polyglutamic acids‡			
α-Poly-L-glutamic acid	Dr. W. E. Hanby	0	Synthetic
α-Poly-L-glutamic acid	Dr. J. Kovács	0	4 Preparations synthesized in different ways
α-Poly-L-glutamic acid	Dr. M. Sela	10	Synthetic: mol wt *ca.* 3,000
α-Poly-L-glutamic acid	Dr. E. R. Blout	2	Synthetic: mol wt *ca.* 90,000
α-Poly-D-glutamic acid	Dr. J. Kovács	0	Synthetic
γ-Poly-DL-glutamic acid	Locally prepared	53	From a culture of *Bacillus subtilis* ATCC strain 9945: mol wt 12,000
γ-Poly-DL-glutamic acid	Dr. M. Bovarnick	50	From a culture of *Bacillus subtilis* ATCC strain 9945: mol wt 12,000
γ-Poly-D-glutamic acid	Dr. J. Kovács	10	From a culture of a thermophilic strain of *Bacillus subtilis*
γ-Poly-D-glutamic acid	Dr. C. B. Thorne	3	From *Bacillus anthracis*
γ-Poly-L-glutamic acid	Dr. J. Kovács	93	Synthetic
Poly-γ-aminobutyric acid	Dr. J. Noguchi	0	Synthetic: mol wt *ca.* 15,200
		breakdown	
Pteroylglutamic acid and p-aminobenzoylglutamic acid peptides§			
Pteroylglutamic acid	Nutritional Biochemicals Corp.	±	Synthetic
Pteroyl-α-diglutamic acid	Dr. H. P. Broquist	−	Synthetic: about 30 per cent pure
Pteroyl-γ-diglutamic acid	Dr. H. P. Broquist	−	Synthetic
Pteroyl-γ,γ-triglutamic acid	Dr. H. P. Broquist	++	Synthetic
Pteroyl-γ-heptaglutamic acid ("Bc conjugate")	Dr. R. D. Greene	++	From yeast: partially purified
p-Aminobenzoyl-L-glutamic acid	Nutritional Biochemicals Corp.	−	Synthetic
p-Aminobenzoyl-γ,γ-triglutamic acid	Dr. H. P. Broquist	+	Synthetic; not completely soluble (purity ?)
p-Aminobenzoylpolyglutamic acid	Dr. S. Ratner	++	From yeast partially purified

TABLE 3—*Continued*

Compound	Source	Breakdown*	Remarks
Proteins¶			
Casein	Nutritional Biochemicals Corp.	−	
Hemoglobin	Nutritional Biochemicals Corp.	−	
Gelatin	Difco Labs., Inc.	−	

* ±, +, ++, Degrees of intensity of ninhydrin spot on the chromatogram; −, no hydrolysis.

† Reaction mixture contained 5 μmoles substrate, 0.2 mg enzyme protein, and M/30 Veronal buffer, pH 8.5, in a total volume of 0.2 ml. Incubation for 5 hr at 37 C. Hydrolysis determined chromatographically, using a butanol-acetic acid-water solution as the solvent and spraying with ninhydrin.

‡ Reaction mixture contained 1 mg polypeptide, 1 mg enzyme protein and M/30 Veronal buffer, pH 8.5, in a total volume of 1.0 ml. Incubation for 6 hr at 37 C. Hydrolysis determined by the ninhydrin color procedure.

§ Reaction mixture as described for peptides. In the case of pteroyl-γ-heptaglutamic acid, 1 mg of the compound was used. Hydrolysis determined chromatographically. The chromatogram of the pteroylglutamic acids was developed for 16 hr in butanol-acetic acid-water and sprayed with ninhydrin; that of *p*-aminobenzoylglutamic acids was developed in phenol-water and sprayed first with Ehrlich's reagent and then with ninhydrin.

¶ Reaction mixture contained 10 mg substrate, 3 mg enzyme protein, and M/30 Veronal buffer, pH 8.5, in a total volume of 3.0 ml. Incubation for 6 hr at 37 C. Hydrolysis of casein and hemoglobin was determined spectrophotometrically in the trichloracetic acid filtrate, using the Beckman model DU spectrophotometer at 280 mμ as described by Northrop *et at.*, (1948), and also by the ninhydrin method. Gelatin hydrolysis was followed by the ninhydrin procedure.

to 46 to 49 per cent of the total glutamic acid present in the polypeptide.

Effect of enzyme and substrate concentrations. As illustrated in figures 2 and 3, in experiments of short duration (10 min), there is a linear relationship between the amount of polyglutamic acid hydrolyzed and enzyme or substrate concentrations over a fairly wide range.

pH Optimum. Figure 4 shows that the optimal pH for the hydrolysis of polyglutamic acid lies between 8.0 and 8.5.

Temperature optimum. As shown in figure 5, the optimal temperature for the hydrolysis of polyglutamic acid is between 35 and 40 C.

Effect of metal ions and inhibitors. The effect of metal ions and inhibitors was tested in a reaction mixture containing 0.33 mg polyglutamic acid, 1.0 mg enzyme protein, and M/30 Veronal buffer, pH 8.5, in a total volume of 1.0 ml. The various metals (as their chlorides) and inhibitors were added in a final concentration of 10^{-2} M or 10^{-3} M. The incubation was carried out for 90 min at 37 C.

None of the following metal ions showed any effect: Mn^{++}, Mg^{++}, Ca^{++} (10^{-2} M); Cu^{++}, Zn^{++}, and Co^{++} (10^{-3} M). None of the following was inhibitory; arsenite; azide; cyanide (10^{-2} M); *p*-chloromercuribenzoate; diethyldithiocarbamate (10^{-3} M); di*iso*propylfluorophosphate (DFP) (10^{-4} M, tested also after preincubation for 2 hr); fluoride (10^{-2} M); 8-hydroxyquinoline (10^{-3} M); iodoacetate (10^{-2} M); and Versene (0.5×10^{-2} M).

Hydrolysis of DNP-polyglutamic acid. In order to ascertain whether terminal amino groups in the polypeptide are essential for the enzymatic hydrolysis, experiments were carried out with the dinitrophenylated polypeptide. The incubation mixture consisted of 2 mg DNP-polyglutamic acid, 2 mg enzyme protein, M/30 Veronal buffer, pH 8.5, in a total volume of 1.5 ml; it was incubated for 5 hr at 37 C. The colorimetric ninhydrin determination showed that 51 per cent of the polypeptide was hydrolyzed, as was the case with the nonsubstituted polypeptide.

Substrate specificity. Various types of peptides were tested as possible substrates for the bacterial enzyme. Peptides used and results are presented in table 3.

With two exceptions, all the α and γ di- and tripeptides were hydrolyzed to their corresponding amino acids, indicating the presence of pepti-

dase in the purified enzyme preparation. The two exceptions, glutathione and γ-L-glutamyl-cysteine were, however, split by the crude enzyme.

Of a number of different compounds designated as α-poly-L-glutamic acid, only one was hydrolyzed to any extent by the purified enzyme, and α-poly-D-glutamic acid was not attacked. Of the γ-polypeptides, the synthetic γ-poly-L-glutamic acid was completely split. The native γ-poly-DL-glutamic acids were hydrolyzed in amounts corresponding to their L-glutamic acid content. With the γ-poly-D-glutamic acid from *B. anthracis*, hydrolysis was negligible, whereas the γ-poly-D-glutamate from the thermophilic strain of *B. subtilis* was partly split. Here, also, the amount of the glutamic acid released corresponded to the L-glutamic acid content of the polypeptide.

With the pteroylglutamyl peptides, the dipeptides were not attacked, while the tripeptide was hydrolyzed with the release of 0.9 moles of glutamic acid per mole of substrate. Glutamic acid was also released from pteroyl-γ-heptaglutamate and *p*-aminobenzoyltri- and polyglutamates, but no quantitative data were obtained, owing to the impurity or incomplete solubility of the substrates.

No hydrolysis could be detected in the three proteins tested.

Hydrolysis of polyglutamic acid by conjugase. The ability of the bacterial enzyme to split pteroyl-γ,γ-tri- and heptaglutamate, and *p*-aminobenzoyltriglutamate, indicates that this enzyme is similar in this respect to chicken pancreas conjugase, known to split these substrates (Mims and Laskowski, 1945; Stokstad, 1954). It was therefore of interest to see whether chicken pancreas conjugase can split the polyglutamate also.

An acetone powder of chicken pancreas was extracted with 7 vol of cold 0.1 M phosphate buffer, pH 7.2, in a Potter-Elvehjem glass homogenizer. The insoluble material was removed by centrifugation for 15 min at 23,000 × G, and the supernatant fluid, containing 50 mg protein per ml, was used as the enzyme source.

Table 4 shows that while both enzymes were able to split pteroyl-γ,γ-triglutamic acid, they differed markedly in ability to hydrolyze γ-poly-DL-glutamic acid.

TABLE 4

Hydrolysis of pteroyl-γ,γ-triglutamic acid and γ-poly-DL-glutamic acid by chicken pancreas conjugase and the bacterial enzyme

Substrate	Enzyme	
	Chicken-pancreas conjugase hydrolysis*	Bacterial enzyme hydrolysis*
	%	%
Pteroyl-γ,γ-triglutamic acid	68	90
γ-Poly-DL-glutamic acid	17	100

* *Pteroyltriglutamate:* 100 per cent hydrolysis corresponds to release of 1 mole glutamic acid per mole substrate (Dabrowska *et al.*, 1949). *Polyglutamate:* 100 per cent hydrolysis corresponds to release of total content of L-glutamic acid.

Conjugase. Reaction mixture contained 2.9 mg (4 μ moles) pteroyltriglutamate or 2 mg polyglutamate, 5 mg enzyme protein, 0.1 M phosphate buffer, pH 7.2, in a total volume of 1.0 ml. Incubation for 15 hr at 37 C. L-Glutamic acid determined manometrically.

Bacterial enzyme. Reaction mixture contained 7.2 mg (10 μ moles) pteroyltriglutamate or 1 mg polyglutamate, 1 mg enzyme protein, M/30, Veronal buffer, pH 8.5, in a total volume of 1.0 ml. Incubation for 4 hr at 37 C. Hydrolysis determined by the ninhydrin procedure.

DISCUSSION

The specificity experiments show that the enzyme obtained from *F. polyglutamicum* n.sp. splits the γ-linked glutamic acid residues in the glutamyl polypeptide, and is thus similar to certain other enzymes known to split the γ-glutamyl bond.

Like the enzyme system found in human red blood cells and tissues (Kream *et al.*, 1954), the bacterial enzyme attacks the L-configuration only; in this respect, both differ from the enzyme produced by *B. subtilis* (Thorne *et al.*, 1954) which preferentially hydrolyzes the poly-D-glutamic acid. On the other hand, the enzymes described by Kream *et al.*, and by Thorne *et al.*, form small quantities of short peptides, while the *F. polyglutamicum* enzyme does not. These peptides, however, may have been formed by a secondary transpeptidation reaction (Williams *et al.*, 1955).

The bacterial enzyme resembles, also, the chicken pancreas conjugase in that both split the γ-peptides of pteroylglutamic acid, and both require a minimum of three terminal glutamic acid residues (Dabrowska *et al.*, 1949) for this

reaction. Furthermore, neither enzyme hydrolyzes glutathione, as does that described by Hird and Springell (1954). As compared with the bacterial enzyme, however, the conjugase splits poly-DL-glutamic acid only at a very slow rate.

The bacterial enzyme categorically differs from conjugase in the following properties: it does not require the calcium ion for activation (Mims and Laskowski, 1945); and it hydrolyzes *p*-aminobenzoylpolyglutamic acid, derived from yeast, which inhibits the conjugase (Sims and Totter, 1947).

Because the bacterial enzyme preparation showed peptidase activity, no final conclusion can be drawn as to whether a single enzyme or several are responsible for the hydrolysis of the γ-glutamyl polypeptide. However, a mechanism by which the reaction might proceed is suggested by the following observations: intact N-terminal groups in the polypeptide are not essential for the attack; DFP, known to inhibit endopeptidases (Jansen *et al.*, 1949), is not inhibitory in this case; the glutamic acid residues are split from pteroylglutamic acid peptides; and poly-γ-aminobutyric acid is not attacked. Hence, it appears likely that the enzyme requires a terminal glutamic acid residue and that it successively removes single amino acids from the carboxyl end of the peptide chain; i. e., that it is a carboxypeptidase. Owing to the presence of dipeptidase activity in the enzyme preparation, however, it cannot at present be surely classified as a γ-glutamyl carboxypeptidase.

Since both the bacterial enzyme and the three other enzymes mentioned above are able to hydrolyze the γ-glutamyl peptide bond, it would seem justifiable to classify them as "γ-glutamyl peptidase" (Kazenko and Laskowski, 1948).

The bacterial enzyme has served to confirm the validity of the γ-poly-L-glutamic acid synthesis (Bruckner *et al.*, 1955*a*), and it appears likely that it can, as well, provide a rapid and specific method for identifying the γ-L-glutamyl bonds in a polypeptide.

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SUMMARY

An organism, *Flavobacterium polyglutamicum* n. sp., which utilizes γ-poly-DL-glutamic acid produced by *Bacillus subtilis*, was isolated from soil. The enzyme which hydrolyzes the polypeptide was obtained from the bacterial cells and purified 10-fold. It specifically splits the γ-linked glutamic acid of the L-configuration in the polypeptide. In addition, the enzyme is able to split glutamic acid residues in pteroly-γ,γ-triglutamate, pteroyl-γ-heptaglutamate, *p*-aminobenzoyl tri- and polyglutamic acids. The nature of the enzymatic hydrolysis is discussed, and it is tentatively suggested that the bacterial enzyme be included in a group, "γ-glutamyl peptidase." The enzyme might be used to detect γ-L-glutamyl residues in a polypeptide.

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