Note

Effect of Glycine on Extracellular Production of Glutamate Oxaloacetate Transaminase and Glutamate Pyruvate Transaminase

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The influence of glycine on extracellular accumulation of protein^{1,2)} and RNA,³⁾ and protoplast preparation⁴⁾ was reported. But there has been no report of a glycine effect on extracellular accumulation of intracellular enzymes. Usually it is troublesome to obtain and purify bacterial intracellular enzymes. We successfully released glutamate oxaloacetate transaminase (GOT, E.C. 2.6.1.1) and glutamate pyruvate transaminase (GPT, E.C. 2.6.1.2) from cells by the addition of glycine to the culture medium. GOT and GPT are intracellular enzymes that are thought to be distributed widely.

The standard medium used was (g/liter): glucose, soluble starch or lactose, 15; polypeptone, 5; yeast extract, 5; K_2HPO_4 , 1.0; $MgSO_4 \cdot 7H_2O$, 0.2; and $MnSO_4 \cdot (4/5) H_2O$, 0.02. If necessary, meat extract, 3; or Na_2CO_3 , 10 (for alkalophilic bacilli) was added. Na_2CO_3 was sterilized separately. Glycine was added to concentrations of from 0.1% to 5%, respectively. Microorganisms were cultured with 50 ml of medium in 300 ml Erlenmeyer, two-tucked or four-tucked flasks (two indentations in the side or four indentations in the side and bottom of an Erlenmeyer flask) after inoculation of an overnight or two day-culture at 30°C or 37°C on a rotary shaker (200 rpm/min). The initial pH was 7.1 (for neutrophilic bacteria), 5.7 adjusted with HCl (for *Acetobacter pasteurianus* and *Gluconobacter roseus*) or 10.3 (for alkalophilic bacilli). GOT and GPT were measured using the reagent from Wako Junyaku Kogyo Co., Ltd. Their activities were assayed by estimating the amount of 2,4-dinitrophenylhydrazone produced from 2,4-dinitrophenylhydrazine and pyruvic acid which was produced by GOT (oxaloacetate is very unstable and turns to pyruvic acid) and GPT. The standard reaction mixture contained 0.05 ml of enzyme solution and 0.2 ml of substrate solution (for GOT, 4 mM α -ketoglutaric acid, 200 mM L-aspartic acid and 0.1 M potassium phosphate buffer, pH 7.4; for GPT, 2 mM α -ketoglutaric acid, 200 mM DL-alanine and 0.1 M potassium phosphate buffer, pH 7.4). The reaction mixture was incubated at 40°C for 15 or 30 min, and then 0.1 ml of 8 mM 2,4-dinitrophenylhydrazine in 5% acetic acid and 95% dimethylformamide was added, followed by incubation at 40°C for 10 min. The

reaction mixture was warmed at 40°C for 10 min with the addition of 0.4 ml of 0.5 M NaOH solution, and the optical density at 520 nm was measured. One unit of enzyme activity was defined as the amount of enzyme which released 1 μ mol of pyruvic acid per min under these conditions. Extracellular protein was obtained by the method of Udaka⁵⁾ and estimated by the method of Lowry *et al.*⁶⁾

As shown in Table I, several strains of *Enterobacteriaceae* and alkalophilic bacilli, *Aeromonas liquefacience*, *Pseudomonas aeruginosa* and *Agrobacterium radiobacter*, showed considerable enzyme excretion on addition of glycine. The GOT or GPT activities which are not listed in the table were lower than 3.0 units per 100 ml culture fluid, though extracellular activities on cultivation with glycine were usually higher than those on cultivation without glycine. The higher the extracellular enzyme productivity, the greater the extracellular protein accumulation was generally. In *P. aeruginosa*, *Rhizobium meliloti*, *Microbacterium lacticum* and *Mycobacterium rhodochrous*, which accumulated more protein on cultivation without glycine than on cultivation with glycine, extracellular protein found on cultivation without glycine was presumably due to cell autolysis, but enzyme activity in the absence of glycine was lower or absent. This suggests that glycine protects the enzyme activity through repression of protease. There were some strains which accumulated high levels of extracellular protein on cultivation with glycine but which showed low level enzyme activity. The enzymes of these strains may be unstable. The sensitivity of *A. radiobacter* to glycine was very different from that of *Serratia marcescens*. This suggests that the inhibitory effect of glycine on cell membrane synthesis and/or cell wall synthesis was strictly different. Strominger and Birge,⁷⁾ and Hammes *et al.*⁸⁾ have reported the inhibitory effect of glycine on cell wall synthesis. There were several other strains which showed decreased growth and did not accumulate protein and enzymes in the presence of glycine. The growth of these strains would be reduced by a different inhibitory mechanism from that in the case of strains which accumulated a considerable amount of protein extracellularly.

Some other cellular enzymes were also released on cultivation with glycine. Alkaline phosphatase and β -galactosidase from *Escherichia coli*, β -galactosidase from alkalophilic *Bacillus* No. C-125 and α -glucosidase from alkalophilic *Bacillus* No. A-59¹²⁾ were accumulated extracellularly more than 20 times as compared with in the case of cultivation without glycine. Further studies on the excretion of these enzymes are in progress.

Table II shows the total activity (intracellular plus extracellular) of GOT. The total activity increased with addition of glycine. *E. coli*, *A. liquefaciens*, *Bacillus* No. 221 and *Bacillus* No. A-40-2 showed good results. *A. liquefaciens* on cultivation with glycine showed more than 11 times the total activity seen on cultivation without glycine, and intracellular enzyme synthesis was activated

TABLE I. EXTRACELLULAR PRODUCTION OF GLUTAMATE OXALOACETATE TRANSAMINASE AND GLUTAMATE PYRUVATE TRANSAMINASE

Strain	Medium	Glycine (%)	Flask	Cultivation time (hr)	Growth (OD ₆₀₀)	GOT or GPT	Activity (units/100 ml)	Protein (mg/ml)
<i>Escherichia coli</i> ^a	L	0	2	24	5.47	GOT	0	0.04
		1.0			3.16		32.2	1.69
		0			5.47	GPT	0.3	0.04
		1.0			3.16		2.2	1.69
<i>Klebsiella pneumoniae</i> IAM 1063	SS	0	2	24	13.08	GOT	3.1	0.17
		1.0			5.84		21.0	1.67
		0			13.08	GPT	0	0.17
		1.0			5.84		7.6	1.67
<i>Enterobacter aerogenes</i> IAM 1183	SS	0	Er	24	12.35	GOT	0	0.10
		2.0			4.62		21.1	1.06
		0			12.35	GPT	0.2	0.10
		2.0			4.62		5.5	1.06
<i>Serratia marcescens</i> IAM 12142	G	0	Er	24	7.58	GOT ^b	0.2	0.12
		5.0			5.82		13.5	1.49
		0			7.58	GPT ^b	1.2	0.12
		5.0			5.82		11.2	1.49
<i>Erwinia herbicola</i> IAM 1562	G	0	Er	24	3.95	GOT	0.2	0.03
		2.0			7.98		5.8	0.18
		0			3.95	GPT	0	0.03
		2.0			7.98		1.2	0.18
<i>Aeromonas liquefaciens</i> IAM 12337	G	0	4	24	13.17	GOT	1.9	0.05
		1.0			10.08		33.5	1.43
		0			13.17	GPT	0.3	0.05
		1.0			10.08		7.6	1.43
<i>Flavobacterium lutescens</i> IAM 1667	SS,ME	0	2	48	3.72	GPT	0	0.13
		0.8			0.36		3.0	0.24
<i>Pseudomonas aeruginosa</i> IAM 1275	G	0	Er	24	12.83	GOT	1.0	0.13
		1.5			13.23		10.1	0.08
<i>Gluconobacter roseus</i> IAM 1839	G	0	2	72	8.57	GPT	0	0.05
		0.1			5.61		0.8	0.07
<i>Acetobacter pasteurianus</i> IAM 1824	G,ME	0	Er	72	4.58	GPT	1.0	0.11
		1.0			0.36		2.2	0.16
<i>Rhizobium meliloti</i> IAM 12035	SS	0	Er	72	6.03	GOT	0.1	0.12
		0.2			1.32		2.1	0.11
<i>Agrobacterium radiobacter</i> IAM 12048	G	0	Er	24	12.43	GOT	5.0	1.09
		0.1			14.23		19.2	2.01
<i>Comamonas terrigena</i> IAM 12052	G,ME	0	2	24	3.72	GOT	0	0.12
		0.3			1.53		1.3	0.46
<i>Micrococcus luteus</i> IAM 12005	G	0	4	24	30.18	GOT	0	0.21
		3.0			0.88		0.8	0.37
<i>Staphylococcus epidermidis</i> IAM 12012	G	0	Er	24	13.67	GPT	0	0.02
		0.5			8.36		0.8	0.04
<i>Bacillus subtilis</i> ^c	G	0	4	24	15.27	GOT	0.7	0.06
		1.5			21.84		3.8	0.12
<i>Corynebacterium fasciens</i> IAM 1079	G,ME	0	2	72	29.55	GOT	0.5	0.28
		1.0			34.34		1.4	0.57
<i>Arthrobacter tumescens</i> IAM 12345	G	0	Er	48	26.57	GOT	0	0.27
		0.3			13.50		2.6	0.27
<i>Cellulomonas fimi</i> IAM 12107	SS	0	2	24	14.47	GOT	0.4	0.05
		0.3			11.11		1.7	1.15
<i>Microbacterium lacticum</i> IAM 1640	G,ME	0	4	24	5.52	GPT	0.6	0.06
		0.3			4.66		2.8	0.03
<i>Mycobacterium rhodochrous</i> IAM 12125	G	0	Er	24	10.04	GOT	0	0.26
		1.5			3.26		0.8	0.10

TABLE I. (Continued)

Strain	Medium	Glycine (%)	Flask	Cultivation time (hr)	Growth (OD ₆₀₀)	GOT or GPT	Activity (units/100 ml)	Protein (mg/ml)
Alkalophilic <i>Bacillus</i> No. 221 ⁹⁾	SS	0	4	24	15.29	GOT	8.6	0.79
		0.5			18.67		21.6	0.89
Alkalophilic <i>Bacillus</i> No. A-40-2 ¹⁰⁾		0	4	24	18.21	GOT	4.6	0.41
		0.5			1.64		21.3	2.22
Alkalophilic <i>Bacillus</i> No. C-125 ¹¹⁾	L	0	4	24	14.72	GOT	0.3	0.62
		1.0			11.59		4.3	0.81

Microorganism were cultured at 30°C except for *Bacillus subtilis* and alkalophilic bacilli which were cultured at 37°C. Erlenmeyer flasks (Er), two-tucked flasks (2) or four-tucked flasks (4) were used. Glucose (G), soluble starch (SS) or lactose (L) was used as the carbon source, and meat extract (ME) was also added if necessary. Other components of the medium, cultivation conditions, the enzyme assay method and the protein determination were described in the text. Protein in the medium precipitated with 5% TCA was 0.070 mg/ml and that in the medium containing meat extract was 0.104 mg/ml.

^a *E. coli* K 12 HB 101 (Leu⁻, Pro⁻, Thiamine⁻ and Str^r).

^b Enzymes were measured after dialysis for 16 hr.

^c *Bacillus subtilis* Marburg 168 GSY 1026 (Tyr⁻, Met⁻).

TABLE II. ENHANCEMENT OF GOT PRODUCTION ON CULTIVATION WITH GLYCINE

Strain	Medium	Glycine (%)	Growth (OD ₆₀₀)	Extracellular activity (units)	Intracellular activity (units)	Total activity (units)
<i>Escherichia coli</i>	L	0	5.47	0	19.3	19.3
		1.0	3.16	32.0	12.0	44.0
<i>Aeromonas liquefaciens</i> IAM 12337	G	0	13.19	1.6	2.4	4.0
		1.0	6.34	35.9	9.4	45.3
<i>Agrobacterium radiobacter</i> IAM 12048	G	0	11.11	3.8	45.7	49.5
		0.1	18.74	17.3	19.5	36.8
<i>Bacillus subtilis</i>	G	0	12.14	0.7	1.2	1.9
		1.5	11.91	3.8	1.4	5.2
Alkalophilic <i>Bacillus</i> No. 221	SS	0	15.29	8.6	39.3	47.9
		0.5	18.67	21.6	43.0	64.6
Alkalophilic <i>Bacillus</i> No. A-40-2	G	0	18.21	4.6	6.5	11.1
		0.5	1.64	21.3	1.1	22.4

For the intracellular enzyme assay, the cell paste was washed and suspended in distilled water containing 2 mM mercaptoethanol. The cell suspension was sonicated for an appropriate time (2~10 min) with cooling. It was confirmed that more than 95% of the cells were disrupted under a microscope. The activity of the supernatant after centrifugation (15,000 *g* × 30 min) was measured. The cultivation conditions and enzyme assay method were the same as in Table I. Activity is expressed at units per 100 ml culture.

directly by addition of glycine because of the high intracellular activity and lower growth. But in *A. radiobacter*, intracellular enzyme synthesis was repressed in the presence of glycine. Although other L-amino acids were examined in the same way, all of them had little effect on enzyme excretion by *E. coli* (data not shown). Glycine made the cell surface permeable to the enzymes, resulting in increased production. Miyashiro *et al.* showed that peptidoglycan components of cell walls on cultivation with glycine were different from those of cell walls on cultivation without glycine in *Bacillus brevis*.²⁾

The cultivation method involving glycine is a useful technique for collecting intracellular enzymes extracellularly compared to autolysis. As our results show, cellular autolysis is not the major cause of enzyme excretion. That enzyme accumulation was not due to cell lysis was shown by the enhancement of total activity, the time course of enzyme production, *etc.* These will be described in subsequent reports.

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