Metabolism of α -Ketoaldehydes in Yeasts: Purification and Characterization of Glyoxalase II from *Saccharomyces cerevisiae*

Kousaku MURATA, Yoshiharu INOUE, Kunihiro WATANABE,
Yasuki FUKUDA, Toshihiko SAIKUSA, Makoto SHIMOSAKA
and Akira KIMURA

*Research Institute for Food Science, Kyoto University,
Uji, Kyoto 611, Japan*

Received July 3, 1985

Glyoxalase II, that catalyzes the conversion of S-lactoylglutathione into lactate and glutathione, was isolated from the yeast, *Saccharomyces cerevisiae*, and some properties of the enzyme were determined. The glyoxalase II was purified approximately 100 fold from a cell extract with a 20% activity yield. Estimation of the molecular weight of the enzyme by both gel filtration and SDS/polyacrylamide gel electrophoresis gave a value of 1.9×10^4 . Among the various thiol esters tested, the enzyme hydrolyzed only S-lactoylglutathione with a $K_m = 7.0 \times 10^{-6}$ M. The enzyme was most active at low pH (pH 3~4) and was stable even in 0.001 N HCl at 40°C for at least 15 min. The activity of the enzyme was inhibited by various thiol compounds such as glutathione and coenzyme A. Hemimercaptal, a non-enzymatic condensation product of methylglyoxal and glutathione, strongly inhibited the activity of the enzyme.

Methylglyoxal, a toxic compound for yeast proliferation, has been thought to be converted into lactate by sequential enzyme reactions catalyzed by glyoxalase I [EC 4.4.1.5] and glyoxalase II [EC 3.1.2.6] in the presence of glutathione (Eq. 1). Yeast glyoxalase I has been purified from *Saccharomyces cerevisiae*

and its properties were determined by Racker¹⁾ and Marmstål *et al.*²⁾ However, attempts to purify glyoxalase II from yeast cells have not been made so far and, therefore, the physiological significance of glyoxalase II and other thiol esterases in yeast cells remains obscure.

(Eq. 1)

In the present communication, 100 fold purification of yeast glyoxalase II and some of its properties are described in comparison with those of glyoxalase II partially purified from human liver by Uotila.³⁾

MATERIALS AND METHODS

Chemicals. S-Lactoylglutathione and the other glutathione thiol esters, except for S-acetylglutathione, were from Sigma Chemical Co., St. Louis, Mo, U.S.A. S-Acetylglutathione was prepared by the method of Kielley

and Bradley.⁴⁾

Cultivation and preparation of a cell extract. Cells of *Saccharomyces cerevisiae* DKD-5D-H (a leu2~3 leu2~112 trp1 his3), which was obtained from Dr. Y. Oshima, were cultured in a medium containing 2.0% yeast extract, 2.0% peptone and 2.0% glucose (pH 5.0, 1.5 liter of medium in a 2 liter Sakaguchi flask) for 24 hr at 30°C with reciprocal shaking. The cells (98 g as wet wt.) from 10 liters of culture fluid were washed once with 0.85% saline solution, resuspended in 10 mM Tris (hydroxymethyl)-aminomethane (Tris)-HCl buffer (pH 7.3) containing 0.1 mM phenylmethylsulfonylfluoride (PMSF) [Tris-PMSF], and then homogenized in a Dyno-Mill for 3 min at 0°C. The homogenate was centrifuged at 25,000 × g for 30 min, and the resultant supernatant was used for the purification of glyoxalase II. Other procedures involved in the cultivation of cells and preparation of cell extracts were described in detail in our previous paper.⁵⁾ Unless otherwise noted, all the purification procedures were performed at 4°C.

Assay for glyoxalase II. Glyoxalase II activity was assayed by following the decrease in absorbancy at 240 nm as described by Uotila.²⁾ The reaction was carried out in a mixture (1.0 ml) containing 1.0 mM S-lactoylglutathione, 50 mM Tris-HCl buffer (pH 7.3) and an appropriate amount of enzyme at 25°C, and the decrease in absorbancy at 240 nm was recorded with a Hitachi Spectrophotometer Model 100-50. At 240 nm, a molar absorption coefficient of 33,000 cm⁻¹ was used for S-lactoylglutathione.¹⁾ Specific activity was expressed as nmol of S-lactoylglutathione hydrolyzed per min per mg of protein. Protein was determined by the method of Lowry *et al.*⁶⁾

Purification of glyoxalase II

(1) **DEAE-cellulose column chromatography.** The supernatant obtained above (5,830 mg protein, 370 ml) was directly applied to a DEAE-cellulose column (5.5 cm × 50 cm) equilibrated with Tris-PMSF, followed by elution with a linear gradient of KCl (0~1.0 M in Tris-PMSF; total volume, 3,000 ml). Fractions were collected at 20 ml/tube/10 min. The active fractions (conductivity, 15~25 mΩ) were pooled, and then concentrated to 50 ml by ultrafiltration with an Amicon PM10 membrane.

(2) **Sephadex G-150 column chromatography.** The active fractions (2,160 mg protein, 480 ml) from the DEAE-cellulose column were applied to a Sephadex G-150 column (4.0 cm × 90 cm) equilibrated with Tris-PMSF, and the enzyme was eluted with Tris-PMSF at a flow rate of 10 ml/tube/15 min. The active fractions (Fr. Nos. 110~155), which were eluted at $V_e/V_o = 2.4$, were combined and concentrated to 20 ml with an Amicon PM10 membrane. V_e and V_o are the elution volume of the enzyme and the void volume of the column, respectively.

(3) **DEAE-Sepharose CL-6B column chromatography.** The enzyme solution (576 mg protein, 20 ml) was applied

to a DEAE-Sepharose CL-6B column equilibrated with Tris-PMSF, and then the enzyme was eluted with a linear concentration gradient of KCl from 0 to 1.0 M (total volume, 1,000 ml). Fractions were collected at 6 ml/tube/12 min, and the active fractions (conductivity, 17~20 mΩ) were pooled, concentrated with an Amicon PM10 membrane, and then dialyzed against 10 mM potassium phosphate buffer (pH 7.3) at 4°C overnight.

(4) **Hydroxylapatite column chromatography.** The dialyzed (88.3 mg protein, 15.7 ml) was applied to a hydroxylapatite column (2.0 cm × 15 cm) equilibrated with 10 mM potassium phosphate buffer (pH 7.3), and then the enzyme was eluted with a linear gradient of the buffer (10~500 mM, total volume, 150 ml). Fractions were collected at a flow rate of 2.5 ml/tube/10 min. The active fractions (conductivity, 27.5~30 mΩ) were combined and concentrated to approximately 2 ml with an Amicon PM10 membrane.

(5) **Sephadex G-75 column chromatography.** The enzyme solution (21.6 mg protein, 2 ml) was chromatographed on a Sephadex G-75 column (1.2 cm × 90 cm) equilibrated with Tris-PMSF at a flow rate of 1.5 ml/tube/15 min. The active fractions (Fr. Nos. 80~95) were pooled, concentrated with an Amicon PM10 membrane, and then dialyzed against 5.0 mM Tris-HCl buffer (pH 7.3) at 4°C overnight. The final enzyme preparation was divided into small portions and stored at -20°C until use.

Molecular weight determination. Molecular weight (M.W.) determination was performed with a calibrated column of Sephadex G-150 (1.0 cm × 90 cm) according to the method of Andrews.⁷⁾ All the proteins were eluted with 5.0 mM Tris-HCl buffer (pH 7.5).

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis in the presence and absence of sodium dodecyl sulfate (SDS) was conducted according to the method of Laemmli.⁸⁾

RESULTS

Purification of glyoxalase II

The overall purification procedure for glyoxalase II is summarized in Table I. The elution pattern of the enzyme on hydroxylapatite column chromatography is shown in Fig. 1. The enzyme was purified approximately 100 fold from a cell extract with a 20% activity yield. The purified enzyme was found to be homogeneous on SDS/polyacrylamide gel electrophoresis (Fig. 2B, lane 7).

Molecular weight determination

By using a calibrated column of Sephadex

TABLE I. SUMMARY OF PURIFICATION OF GLYOXALASE II

Step	Total protein (mg)	Specific activity (nmol/min/mg-protein)	Total activity (nmol/min)	Yield (%)	Fold
1. Cell extract	5,830	13.8	80,500	100	1.00
2. DEAE-cellulose	2,160	43.4	93,700	116	3.14
3. Sephadex G-150	576	83.5	48,200	59.9	6.05
4. DEAE-Sepharose CL-6B	88.3	340	30,000	37.3	24.6
5. Hydroxylapatite	21.6	1,150	24,800	30.8	83.3
6. Sephadex G-75	11.0	1,340	14,700	18.3	97.1

The purification procedure was described in detail under MATERIALS AND METHODS.

FIG. 1. Elution Pattern of Glyoxalase II on Hydroxylapatite Column Chromatography.

The detailed chromatographic conditions were given under MATERIALS AND METHODS. Elution of the enzyme was started at the position indicated by an arrow. —○—, absorbancy at 280 nm; —●—, activity; ----, conductivity of eluates.

G-150, a molecular weight (M.W.) of 19,000 was obtained for the purified glyoxalase II (Fig. 2A). The enzyme treated with SDS in the presence of dithiothreitol also migrated as a single protein band of a M.W. = 19,000 (Fig. 2B, lane 7).

Effect of pH and temperature

The enzyme was remarkably active at low pH (pH 3~4) (Fig. 3A). Above pH 9.0, the activity was rapidly lost. The enzyme was stable in the broad pH range of 4~8 (Fig. 3B).

The enzyme was most active at 45°C in 10 mM Tris-HCl buffer (pH 7.0) (data not shown). A 50% activity loss occurred on incubation of the enzyme in 10 mM Tris-HCl buffer (pH 7.0) at 40°C for 14 min, and at 50°C for 2 min (Fig. 4). The stability of the enzyme

in 0.001 N HCl at various temperatures was examined (Fig. 4). The enzyme was stable even in 0.001 N HCl at 40°C for at least 15 min.

Inhibitors

The effects of several metal ions on the glyoxalase II activity were investigated (Table II). The enzyme was neither activated nor inhibited by the divalent metal ions tested (Mn^{2+} , Mg^{2+} , Ni^{2+} , Co^{2+} , Ca^{2+} ; 5.0 mM each). Metal chelators such as ethylenediaminetetraacetate (2.5 mM) and 8-hydroxyquinoline (2.5 mM) also had no effect. Reducing agents inhibited the activity of the enzyme. The inhibition was 60% with dithiothreitol at 1.25 mM, and 43% with glutathione at 8.0 mM. *p*-Chloromercuribenzoic acid and $HgCl_2$ completely inhibited the activity at

FIG. 2. Molecular Weight Determination of Glyoxalase II.

A: Sephadex G-150 column chromatography of glyoxalase II. V_e (elution volume)/ V_o (void volume) was plotted *versus* Log [M.W.]. The position of glyoxalase II is shown by a closed circle. The standard proteins used were: a, pyruvate kinase (M.W.=57,000); b, glyceraldehyde-3-phosphate dehydrogenase (M.W.=36,000); c, trypsin (M.W.=23,000); and d, lysozyme (M.W.=14,300).

B: SDS/polyacrylamide gel electrophoresis of samples at various purification steps. Samples at various purification steps were subjected to SDS (0.1%)/polyacrylamide (12.5%) gel electrophoresis (lanes 2~7). The samples and protein amounts applied were: lane 2, cell extract (80 μ g); lane 3, after DEAE-cellulose column chromatography (40 μ g); lane 4, after Sephadex G-150 column chromatography (40 μ g); lane 5, after DEAE-Sephadex CL-6B column chromatography (20 μ g); lane 6, after Hydroxylapatite column chromatography (5 μ g); and lane 7, after Sephadex G-75 column chromatography (5 μ g). The standard proteins used in Lane 1 were: myosin (H-chain) (M.W.=200,000); phosphorylase B (M.W.=97,400); bovine serum albumin (M.W.=68,000); ovalbumin (M.W.=43,000); α -chymotrypsinogen (M.W.=25,700); β -lactoglobulin (M.W.=18,400); and lysozyme (M.W.=14,300).

FIG. 3. Effect of pH on Activity (A) and Stability (B) of Glyoxalase II.

For determination of the optimal pH for activity, the reaction was carried out at various pHs, the activity seen at pH 7.0 (Tris-HCl buffer) being taken as 100%. The buffers used were: —○—, acetate buffer; —●—, Tris-HCl buffer; —□—, glycine-KOH buffer; ●, 0.001 N HCl; and ○, 0.01 N HCl. For determination of the stability of the enzyme, 5.0 μ g of enzyme was incubated at 40°C for 5 min at various pHs, and then residual activity was assayed under the standard conditions. The buffers used were: —○—, acetate buffer; —●—, Tris-HCl buffer; —□—, glycine-KOH buffer; and ●, 0.001 N HCl. The activity at pH 7.0 was taken as 100%.

FIG. 4. Effect of Temperature on Stability of Glyoxalase II.

The enzyme (5.0 μ g) was incubated in 10 mM Tris-HCl buffer (pH 7.0) (-----) or 0.001 N HCl (—) at various temperatures ($\circ$, 25°C; $\circ$, 40°C; $\bullet$, 50°C; and $\bullet$, 60°C) for several minutes, and then residual activities were assayed under the standard conditions.

0.3 mM and 0.5 mM, respectively. Formaldehyde, acetaldehyde, glycoaldehyde and pyruvaldehyde had no effects at 10 mM (data not shown). The effect of hemimercaptal, a non-enzymatic condensation product of methylglyoxal and glutathione, was studied (Table III). When both of reduced glutathione and methylglyoxal were present, marked inhibition of glyoxalase II activity was observed at the concentration at which neither alone was so inhibitory, except for in the case of glutathione at 8.0 mM. The inhibition increases with increasing concentration of either reduced glutathione or methylglyoxal.

Substrate specificity

The enzyme specifically hydrolyzed *S*-lactoylglutathione. *S*-Acetylglutathione, *S*-methylglutathione, *S*-butylglutathione, *S*-(*n*-propyl)glutathione and *S*-hexylglutathione were not hydrolyzed by the enzyme. The substrate (*S*-lactoylglutathione) saturation curve for the glyoxalase II reaction is shown in Fig. 5A. When the results in Fig. 5A were plotted in a double reciprocal form, a parabolic relationship was observed (data not shown). However,

TABLE II. EFFECTS OF VARIOUS CHEMICALS ON GLYOXALASE II ACTIVITY

Chemicals	(mM)	Relative activity (%)
None	—	100
MnCl ₂	5.0	89.3
NiCl ₂	5.0	82.1
CaCl ₂	5.0	96.4
CoCl ₂	5.0	92.9
MgCl ₂	5.0	85.7
HgCl ₂	0.5	0
	0.05	71.4
	0.005	89.3
<hr/>		
Iodoacetamide	0.63	88.9
	1.25	80.0
<i>N</i> -Ethylmaleimide	1.25	102
	2.50	87.8
Ethylenediaminetetraacetate	2.50	111
8-Hydroxyquinoline	2.50	102
<i>p</i> -Chloromercuribenzoate	0.30	0
Dithiothreitol	0.63	65.7
	1.25	38.9
Glutathione	2.0	93.6
	4.0	85.1
	8.0	57.4
Coenzyme A	0.25	20.1
	0.50	0

Activities were determined under the standard conditions in the presence of various chemicals at the concentrations indicated.

this could be converted into a linear relationship by plotting the reciprocal of the square of the substrate concentration (Fig. 5B), and the *K_m* of the enzyme for *S*-lactoylglutathione was estimated to be 7×10^{-6} M. The reaction products of glyoxalase II reaction were determined enzymatically to be lactate and glutathione with lactate dehydrogenase and glutathione reductase (data not shown).

DISCUSSION

Glyoxalase II is one of the thiol esterases and catalyzes the hydrolysis of glutathione thiol esters formed from α -keto-aldehydes (glyoxal, methylglyoxal and phenylglyoxal) and

TABLE III. INHIBITION OF GLYOXALASE II ACTIVITY BY HEMIMERCAPTAL

Assay conditions	Relative activity (%)	Inhibition (%)
Control (No addition)	100	0
Control + 5.0 mM MG	85.1	14.9
+ 10 mM MG	80.8	19.2
+ 20 mM MG	76.6	23.4
Control + 2.0 mM GSH	93.6	6.4
+ 4.0 mM GSH	85.1	14.9
+ 8.0 mM GSH	57.4	42.6
Control + 10 mM MG		
2.0 mM GSH	21.3	78.7
+ 10 mM MG		
4.0 mM GSH	12.8	87.2
+ 10 mM MG		
8.0 mM GSH	1.3	98.7
Control + 4.0 mM GSH		
5.0 mM MG	10.2	89.8
+ 4.0 mM GSH		
10 mM MG	0.8	99.2

Activities were determined under the standard conditions in the presence of methylglyoxal (MG) or glutathione (GSH), or both.

glutathione through the action of glyoxalase I (Eq. 1). Glyoxalase II has been purified from beef liver¹⁾ and human liver,¹⁾ and the properties of the latter enzyme were determined in detail. The yeast glyoxalase II presented here was closely similar to the human liver enzyme in molecular weight (yeast enzyme M.W. = 19,000; human liver enzyme M.W. = 22,000), sensitivity to inhibitors (hemimercaptal and reducing agents such as glutathione) and the pH-activity profile.

The severe inhibition (feedforward inhibition) of glyoxalase II by hemimercaptal, a substrate for glyoxalase I, is of physiological significance in regulation of the level of *S*-lactoylglutathione in yeast cells. The yeast cells used showed no detectable *S*-lactoylglutathione-hydrolyzing activity other than that of glyoxalase II (unpublished data). So, the inhibition of glyoxalase II by hemimercaptal inevitably leads to accumulation of *S*-

FIG. 5. Relationship between Reaction Velocity and Substrate Concentrations.

A: Saturation curve for the glyoxalase II reaction. The reaction was carried out under the standard conditions except that the substrate (*S*-LG: *S*-lactoylglutathione) concentration was varied and the velocity ($\mu\text{mol}/\text{min}/\text{mg}$ -protein) was plotted as a function of *S*-LG concentration. B: Double reciprocal plots of the velocity (arbitrary unit) versus the square of the *S*-LG concentration.

lactoylglutathione in cells. A similar proposal that the function of glyoxalase I and II is to regulate the cellular level of *S*-lactoylglutathione, has been made by Gillespie.⁹⁾ These observations indicate an important function of *S*-lactoylglutathione in cellular metabolism.

In mammalian cells, *S*-lactoylglutathione potentiates histamine release from human leucocytes¹⁰⁾ and modulates microtubule assembly *in vitro*.¹¹⁾ Evidence has also accumulated as to the function of *S*-lactoylglutathione in modulating immune and inflammatory responses. The 12-*O*-tetradecanoylphorbol-13-acetate, with the ability to induce tumors in mouse skin, has been shown to increase the level of *S*-lactoylglutathione in cells by increasing the glyoxalase I activity and decreasing the glyoxalase II activity.⁹⁾ The increased *S*-lactoylglutathione level (*i.e.* a decreased level

TABLE IV. PROPERTIES OF THE ENZYME RESPONSIBLE FOR METHYLGLYOXAL METABOLISM IN YEAST

Properties	Methylglyoxal synthase	Glyoxalase I	Glyoxalase II	Methylglyoxal reductase	Lactaldehyde dehydrogenase
M.W.	26,000	32,000	19,000	43,000 (Glycoprotein)	40,000
Substrate specificity	Specific to DHAP	G, MG, PhG	Specific to S-LG	G, MG, PhG Specific to NADPH	Specific to L-LAL and NAD
<i>K_m</i>	3.0 mM (for DHAP)	0.15 mM (for MG) 0.10 mM (for PhG)	7.0 μM (for S-LG)	5.88 mM (for MG) 1.54 mM (for PhG) 0.20 mM (for NADPH)	10 mM (for L-LAL) 2.9 mM (for NAD)
Optimal pH	9~10	6~8	3~4	7.0	6.5
Inhibitors	3-PGA, PEP, GSH, L-cysteine	EDTA Zn ²⁺	GSH Coenzyme A Hemimercaptal	NADP (<i>K_i</i> = 70 nM)	
Activators		Polyamines Fe ²⁺		GSH, DTT, 2-ME	Halide ion (Cl ⁻ , I ⁻ , Br ⁻)

Abbreviations: DHAP, dihydroxyacetonephosphate; 3-PGA, glyceraldehyde-3-phosphate; PEP, phosphoenolpyruvate; GSH, reduced glutathione; G, glyoxal; MG, methylglyoxal; PhG, phenylglyoxal; S-LG, S-lactoylglutathione; DTT, dithiothreitol; 2-ME, 2-mercaptoethanol; L-LAL, L-lactaldehyde; EDTA, ethylenediaminetetraacetate.

FIG. 6. Tentative Pathway for Methylglyoxal Metabolism (Glycolytic Bypass) in *S. cerevisiae*.

The abbreviations used for intermediates are: Glc, glucose; FBP, fructose-1,6-bisphosphate; DHAP, dihydroxyacetonephosphate; G-3-P, glyceraldehyde-3-phosphate; 1,3-BPG, 1,3-bisphosphoglycerate; 3-PG, 3-phosphoglycerate; 2-PG, 2-phosphoglycerate; PEP, phosphoenolpyruvate; MG, methylglyoxal; L-lactaldehyde; *S*-LG, *S*-lactoylglutathione; AT, acetol (1-hydroxy-2-propanone); LA, lactic acid; Pyr, pyruvate; and GSH, reduced glutathione. Enzymes: 1, methylglyoxal synthase; 2, methylglyoxal reductase; 3, L-lactaldehyde dehydrogenase; 4, glyoxalase I; 5, glyoxalase II; and 6, lactate dehydrogenase. The activity converting MG to pyruvate or AT, of which the pathways are shown by dotted lines was not detected in our yeast strain.

of glyoxalase II activity) is related to the tumor promoting properties of the compound. In fact, Jerzykowski *et al.*^{12,13)} reported that no glyoxalase II activity was detected in approximately two-thirds of the tumors studied, while in the remaining one-third it was low compared to the activity in normal tissues. However, no data have been reported so far as to the function of *S*-lactoylglutathione in microbial cells.

Taking into account the similar properties of the glyoxalase II in yeast and mammalian cells, yeast cells may constitute a rapid and sensitive system for the elucidation of the functions of *S*-lactoylglutathione. For studies in this direction, we have already purified the enzymes responsible for the methylglyoxal metabolism in yeast. These enzymes are methylglyoxal synthase [EC 4.2.99.11]¹⁴⁾, methylglyoxal reductase,⁵⁾ lactaldehyde dehydrogenase¹⁵⁾ and glyoxalase II presented here. The properties of these enzymes are summarized in Table IV. Based on the results of product analyses of the reactions catalyzed by these enzymes, we established, for the first time, the pathway for methylglyoxal metabolism (Glycolytic bypath; Fig. 6). Our preliminary data¹⁶⁾ on the enzyme activities involved in the bypath indicated that the activity converting methylglyoxal to lactate *via* lactaldehyde was high in growing cells and low in non-growing cells. On the other hand, the activity converting methylglyoxal to lactate *via* *S*-lactoylglutathione was low in growing cells and high in non-growing cells. We are now investigating the physiological functions of the two routes leading from methylglyoxal to lactate *via* lactaldehyde or *S*-lactoylglutathione.

The functions of intermediates (methylglyoxal, hemimercaptal, *S*-lactoylglutathione and lactaldehyde) in yeast cell division are also being investigated.

Acknowledgments. We thank Dr. M. Sakaguchi for his continuous help in this work. We also thank Ms. N. Okamoto for her technical assistance. This study was supported in part by a Grant-in-Aid for Scientific Research (No. 60560114) from the Ministry of Education, Science and Culture of Japan.

REFERENCES

- 1) E. Racker, *J. Biol. Chem.*, **190**, 685 (1951).
- 2) E. Marmstal, A.-C. Aronsson and B. Mannervik, *Biochem. J.*, **183**, 23 (1979).
- 3) L. Uotila, *Biochemistry*, **12**, 3944 (1973).
- 4) W. W. Kielley and L. B. Bradley, *J. Biol. Chem.*, **206**, 327 (1954).
- 5) K. Murata, Y. Fukuda, K. Watanabe, M. Shimosaka, T. Saikusa and A. Kimura, *Eur. J. Biochem.*, **151**, 631 (1985).
- 6) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
- 7) P. Andrews, *Biochem. J.*, **96**, 595 (1969).
- 8) U. K. Laemmli, *Nature*, **227**, 135 (1979).
- 9) E. Gillespie, *Biochem. Biophys. Res. Commun.*, **98**, 463 (1981).
- 10) E. Gillespie, *Nature*, **227**, 135 (1979).
- 11) E. Gillespie, *Fedn. Proc.*, **34**, 541 (1975).
- 12) T. Jerzykowski, R. Winter, W. Matsuzewski and Z. Szeziurek, *Experientia*, **31**, 32 (1975).
- 13) R. Winter, K. Piskorska and T. Jerzykowski, *Neoplasma*, **25**, 465 (1978).
- 14) K. Murata, Y. Fukuda, K. Watanabe, T. Saikusa, M. Shimosaka and A. Kimura, *Biochem. Biophys. Res. Commun.*, **131**, 190 (1985).
- 15) Y. Inoue, K. Murata, K. Watanabe, T. Saikusa, M. Shimosaka and A. Kimura, *Eur. J. Biochem.*, in press (1985).
- 16) K. Murata, Y. Inoue, K. Watanabe, Y. Fukuda, T. Saikusa, M. Shimosaka and A. Kimura, *J. Ferment. Technol.*, in press (1985).