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PURIFICATION AND PROPERTIES OF PENICILLIN ACYLASE OF *BOVISTA PLUMBEA*

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Summary

1. A penicillin acylase (penicillin amidohydrolase, EC 3.5.1.11) formed constitutively in the basidiomycete *Bovista plumbea* was purified 220-fold by a combination of two gel filtration runs, ion-exchange chromatography on DEAE-cellulose, ultrafiltration and final chromatography on hydroxyapatite. Recovery was 40%.

2. The enzyme was clearly distinguished from penicillin acylases previously characterized: the molecular weight of the purified enzyme was evaluated by gel filtration to be 88 000. K_m for the best substrate phenoxymethylpenicillin was 1.67 mM. The maximum of activity occurred at 52°C and pH 7.5. The activation energy calculated by Arrhenius' graphic method was 16.45 kJ/mol.

3. Neither 8-hydroxyquinoline nor EDTA, iodoacetic acid, or the products of enzymatic cleavage, 6-aminopenicillanic acid or phenoxyacetic acid, showed any characteristic inhibition effect.

4. The substrate spectrum of the enzyme was elucidated. Phenoxymethylpenicillin was the best substrate. *N*-Acylamino acids, dipeptides, and tripeptides were not hydrolyzed; affinity occurred only towards penicillins lacking a nitrogen atom in the side chain acid. Penicillins with aryloxy residues possessing hydrophilic groups are favoured above aryl residues and short side chains above bulky ones.

Introduction

Penicillin acylase (penicillin amidohydrolase, EC 3.5.1.11) causes the removal of the side chain of penicillins to yield 6-aminopenicillanic acid, the nucleus of the penicillin molecule. Semisynthetic penicillins of improved clinical effec-

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tiveness are available by chemical acylation of 6-aminopenicillanic acid. Thus penicillin acylase is an enzyme of great commercial importance delivering 6-aminopenicillanic acid in amounts large enough to produce semisynthetic penicillins on an industrial scale [1]. Enzymatic activity has been demonstrated in fungi, yeasts, actinomycetes and bacteria. The enzyme has been reviewed by Hamilton-Miller [2], Huber et al. [3], Vandamme and Voets [4], Savidge and Cole [5], and Vanderhaeghe [6]. On the basis of results obtained by investigating mainly intact cells, crude extracts or slightly purified enzyme preparations, three types of penicillin acylase have been distinguished so far: (i) "bacterial acylases", acylases preferably cleaving benzylpenicillin, (ii) "fungal acylases" preferably splitting phenoxymethylpenicillin, and (iii) the recently discovered "ampicillin acylase" [7,8] which is directed towards α-aminobenzylpenicillin (ampicillin).

In most of the cases "fungal acylases" have been described to be enzymes hydrolyzing phenoxymethylpenicillin much better than benzylpenicillin and other penicillins, and "bacterial acylases" to be enzymes with very low activity towards phenoxymethylpenicillin. However, exceptions to this rule are described for *Erwinia aroideae* [9], for *Achromobacter* sp. BRL 1755 (NCIB 9424) [10], and for *Micrococcus ureae* KY 3767 [11]: phenoxymethylpenicillin is a better substrate than benzylpenicillin for the penicillin acylases formed by these bacteria. In addition, "ampicillin acylase" was found in bacteria only. Penicillin acylase activity was observed also in the basidiomycetes *Pleurotus ostreatus* [12] and *Bovista plumbea* [13]. Only few penicillin acylases have been purified to elucidate their substrate spectrum as well as their physical and biochemical characteristics [9,14—19]. The present paper describes the purification and properties of the penicillin acylase produced by *B. plumbea* NRRL 3501. The results obtained support the suggestion that penicillin acylases should not be classified by the type of organism producing them but rather according to the type of penicillin preferably hydrolyzed. The classical "fungal acylases" are in fact part of the group of phenoxymethylpenicillin acylases.

Materials and Methods

Materials. B. plumbea NRRL 3501 mycelium and penicillins used (phenoxymethylpenicillin, benzylpenicillin, *p*-hydroxyphenoxymethylpenicillin, *o*-kresoxymethylpenicillin, thymoxymethylpenicillin, ampicillin, oxacillin and hetacillin) were supplied by Biochemie G.m.b.H. *N*-acylamino acids (*N*-benzoyl, *N*-acetyl, and *N*-phenoxyacetyl derivatives of glycine, L-phenylalanine, L-aspartic acid, *S*-benzoyl-L-cysteine, DL-methionine, DL-alanine, and DL-valine) were prepared as described by Edsall [20]. Dipeptides and tripeptides were purchased from Fluka A.G., Buchs, and Schuchardt, München. Preparation of hydroxyapatite was performed by a method previously described by Atkinson et al. [21]. DEAE-cellulose DE 23 was a product of Whatman Biochemicals Ltd. Various Sephadex gels were obtained from Pharmacia Fine Chemicals. Polyacrylamide gels (P 100 and P 200) were purchased from Bio-Rad-Laboratories.

Determination of protein concentration. Protein was determined by the method of Lowry et al. [22] or spectrophotometrically [23].

Determination of enzymatic activity. (i) Substrate: *N*-acylamino acids and

peptides: An assay described by Roehr et al. [24] measuring the amount of amino acids released by enzymatic cleavage was used. Substrate concentration was 25.6 mM *N*-acyl-L-amino acid or peptide.

(ii) Substrate: penicillins: Enzymatic activity was determined by pH-stat titration of the amount of side chain acid produced from a reaction mixture containing 25.6 mM substrate, and the enzyme preparation kept at 30°C and at pH 7.5 (standard reaction mixture). In both cases a blank with distilled water instead of enzyme solution was carried out to provide for instability of substrates. The titrations were performed with 50 mM NaOH, using the combination ABU 1b/TTT 11/SBR 2c (Radiometer A/S, Kopenhagen).

One unit of enzymatic activity is defined as the amount of enzyme required to produce 1 μmol of 6-aminopenicilianic acid or L-amino acid per min under conditions described above.

The rate of hydrolysis was constant between 0 and 15 units/ml within 30 min.

Results

Preparation of cell free extracts

B. plumbea mycelium had an enzymatic activity of 24.67 units/g dry weight. By extraction of untreated mycelium as well as of acetone-dried mycelium only 21% of the total activity were obtained in the supernatant after centrifuging for 15 min at 10 000 × *g*. Previous homogenization carried out in a mortar at −20°C, in a Potter-Elvehjem homogenizer or by ultrasonication and subsequent extraction with dilute alkali or 10 mM potassium phosphate buffer (pH 7.5, buffer A) released up to 28% of activity. A yield of 51% was obtained by the following method finally used: A suspension of 52.5 g mycelium (dry wt.) per l buffer A was homogenized using an "Ultra Turrax" homogenizer (T 45, Janke and Kunkel K.G., Staufen, G.F.R.). The suspension was further treated by disruption in a Manton-Gaulin homogenizer (type 15 M, APV. Co. Ltd., Crawley, Surrey, U.K.) at a pressure of 800 bar. Flow rate was 0.93 l/min. Due to the heat stability of the enzyme (as demonstrated below) no loss of activity occurred during disruption of the cells.

Protein concentration in the clear yellow-brown supernatant was 7.9 mg/ml with a specific activity of 0.12.

The following operations were carried out at room temperature, if not described otherwise.

Gel filtration on Sephadex G-25 coarse

Gel filtration turned out to be a method quite useful not only for purification but also for introducing the appropriate buffer for a subsequent ion-exchange chromatography. Since fractionation of crude extract on Sephadex G-200 and G-100 as well as precipitation by $(NH_4)_2SO_4$ or by acetone was unsuccessful on account of unfavourable yields, gel filtration on Sephadex G-25 was found to be suitable for the removal of low molecular weight components.

5 ml of the crude extract were placed on a 1.5 × 27-cm column of Sephadex G-25 coarse gel previously equilibrated in buffer A. The column was washed with buffer A at a flow rate of 70 ml/h. 99% of the penicillin acylase activity

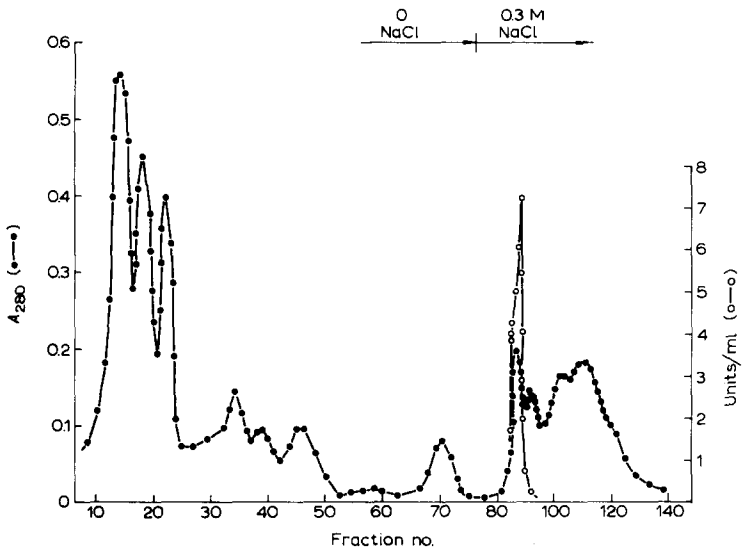

Fig. 1. DEAE-cellulose chromatography of G-25 fraction. Seven G-25 fractions (35 ml containing 1.99 mg protein per ml and a total of 22.34 units of enzyme) were passed through a 2.5 X 39-cm column of DEAE-cellulose equilibrated with buffer A. After washing with four column volumes buffer A, elution was carried out with buffer A which was 0.3 M with respect to NaCl. The column was operated at a flow rate of 34.4 ml/h, volume of fractions was 9.6 ml for elution with buffer A and 2.2 ml after change of buffer. o———o, penicillin acylase, •———•, A_{280nm}.

appeared in the void volume. Specific activity of the pooled active fractions (designated as the G-25 fraction) was 0.32.

DEAE-Cellulose chromatography

Seven G-25 fractions (35 ml containing 69.8 mg protein) were applied to a 2.5 cm X 39 cm column of DEAE-cellulose previously equilibrated with buffer A. The column was washed with four column volumes buffer A. As shown in Fig. 1, large amounts of protein and brown-coloured material were eluted under these conditions. The wash was changed to buffer A which was 0.3 M with respect to NaCl. Enzyme was eluted with the exclusion volume, followed by inactive protein. The fractions of highest specific activities (fractions 85—92) were combined and designated as the DEAE fraction (17.6 ml). The DEAE fraction had a specific activity of 4.12; recovery was 48% with regard to the G-25 fraction. No loss in activity was observed upon storage at −20°C for 3 months. This stepwise elution proved to be superior over applying a gradient from 0 to 0.4 M NaCl over a total volume of 800 ml.

Chromatography on Bio Gel P-200

The DEAE fraction was dialyzed against distilled water for 24 h at 4°C, and the retentate was subsequently concentrated at the same temperature in a Minicon B concentrator to a final volume of 3 ml. The concentrated DEAE fraction (2.62 mg protein, 10.80 units) was placed on a 1.6 cm X 66 cm column of Bio Gel P-200 equilibrated with buffer A which contained 2.2% NaCl. The column was washed with the same buffer at a flow rate of 10.2 ml/h. Some inactive

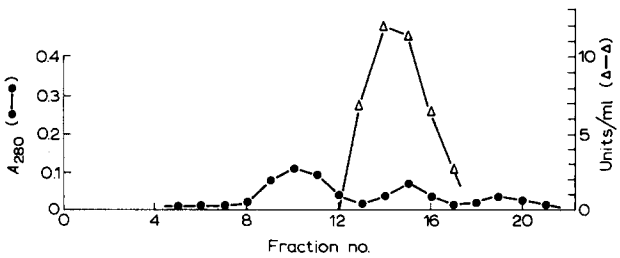

Fig. 2. Hydroxyapatite chromatography of P-200 fraction. The P-200 fraction was dialyzed and concentrated to a final volume of 2 ml with a protein concentration of 0.325 mg/ml and a total of 9.82 units of enzyme. The 1.5 X 30-cm hydroxyapatite column previously equilibrated with 5 mM potassium phosphate (pH 6.8) was washed with the same buffer. 2.3-ml fractions were collected at a flow rate of 12 ml/h. ●———●, A_{280nm}, △———△, penicillin acylase.

protein appeared in the void volume which was well separated from the enzyme protein. 92% of the applied activity were recovered in a total volume of 9.7 ml (P-200 fraction) with a specific activity of 15.17.

Hydroxyapatite chromatography

The P-200 fraction was dialyzed and concentrated as described above to a final volume of 2 ml. The enzyme solution (2 ml with 9.82 units) was applied to a 1.5 X 30-cm column of hydroxyapatite previously equilibrated with 5 mM potassium phosphate (pH 6.8). The column was washed with the same buffer at a flow rate of 12 ml/h. As shown in Fig. 2, enzyme protein was eluted well separated from two other protein components with no enzymatic activity. The combined fractions (Nos. 13—16) yielded a total of 9.05 units at a specific activity of 26.45. Recoveries were usually 90—95%. The enzyme solutions were concentrated to a protein concentration of 0.1 mg/ml and could be frozen and thawed repeatedly with little loss of activity. Table I summarizes the purification procedure. The concentrated hydroxyapatite fraction split 26.45 μmol of phenoxymethylpenicillin per min per mg protein which represented a 220-fold purification of the activity present in the crude extract.

TABLE I

PURIFICATION OF PENICILLIN ACYLASE ACTIVITY OF *B. PLUMBEA*

The crude extract was prepared by disruption of the mycelium by means of a Manton-Gaulin homogenizer and centrifuging of the suspension for 15 min at 10 000 X *g*. 4 ml of the supernatant were passed through a Sephadex G-25 column. The purification was carried out by chromatography of seven G-25 fractions on DEAE-cellulose, followed by gel filtration on Bio-Gel P-200 and final chromatography on hydroxyapatite.

Fraction	Total volume (ml)	Total units	Recovery (%)	Total protein (mg)	Specific activity	Purified (-fold)
Crude extract	28	22.59	100	188.3	0.12	1.00
G-25	35	22.34	99.3	69.8	0.32	2.67
DEAE	18	10.80	47.8	2.62	4.12	34.30
P-200	10	9.86	43.6	0.65	15.17	126.40
Hydroxy-apatite	9	9.05	40.1	0.34	26.45	220.40

Molecular weight determination

Three molecular weight estimations were made using a 2.5 cm × 98 cm Sephadex G-200 superfine column previously equilibrated in buffer A containing 2.2% NaCl. 4 ml of enzyme solution were applied and the elution was carried out at a flow rate of 3.8 ml/h at 4°C with the same buffer. The column was calibrated with cytochrome *c*, chymotrypsinogen A, hen's egg albumin, catalase and ferritin, all obtained from Boehringer.

Two experiments were carried out with two hydroxyapatite fractions. Penicillin acylase activity was located at $K_{av} = 0.35$—0.37 ($K_{av} = (V_e - V_o)/(V_t - V_o)$), corresponding to a molecular weight of 83 000—93 000. The Fusarium semitectum enzyme had a molecular weight of 65 000 [16], penicillin acylases of bacteria had molecular weights in the range from 62 000 to 270 000 [9,14,15,17,19].

Substrate specificity

The activity of the purified enzyme was assayed against three groups of substrates: *N*-acylamino acids, peptides, and penicillins.

(1) N-acylamino acids. The following substrates were tested: *N*-benzoyl-glycine, -L-phenylalanine, - DL-aspartic acid, -*S*-benzoyl-L-cysteine, -DL-methionine, and -DL-alanine; *N*-Acetyl-DL-phenylalanine, -L-aspartic acid, -DL-methionine, and -DL-alanine; *N*-Phenoxyacetyl-glycine, -L-phenylalanine, -DL-aspartic acid, -*S*-benzoyl-L-cysteine, -DL-methionine, -DL-alanine, -DL-valine, and -DL-lysine. No activity was detected with any of these substrates. Thus the penicillin acylase was significantly marked off from other amidohydrolases such as hippurate hydrolase (*N*-benzoylamino acid amidohydrolase, EC 3.5.1.32), aminoacylase (*N*-acylamino acid amidohydrolase, EC 3.5.1.14), and aspartoacylase (*N*-acyl-L-aspartate amidohydrolase, EC 3.5.1.15).

(2) Di- and tripeptides. No cleaving activity was observed with glycyl-glycine, glycyl-L-leucine, benzyloxycarbonylglycyl-L-phenylalanine and glycyl-glycyl-glycine. Thus the enzyme had no carboxypeptidase A (peptidyl-L-amino acid hydrolase, EC 3.4.12.2) activity. Activity of glycyl-glycine dipeptidase (glycyl-glycine hydrolase, EC 3.4.13.1), tripeptide aminopeptidase (α-aminoacyl-dipeptide hydrolase, EC 3.4.11.4) and glycine carboxypeptidase (peptidyl-glycine hydrolase, EC 3.4.12.8) could also not be detected in the enzyme preparation.

(3) Penicillins. B. plumbea penicillin acylase was found to be specific for the cleavage of phenoxymethylpenicillin, *p*-hydroxyphenoxymethylpenicillin, *o*-kresoxymethylpenicillin, benzylpenicillin and thymoxymethylpenicillin. The specific activities of a hydroxyapatite fraction towards penicillins are summarized in Table II.

Evaluation of K_m and activation energy

Only few data concerning kinetic properties of purified penicillin acylases are published. K_m was 4.5 mM benzylpenicillin for the enzyme of *Bacillus megaterium* [14], 3.7 mM cephalexin for the penicillin acylase of *Xanthomonas citri* IFO 3835 [17], 1.4 mM cephalexin for the enzyme of *Kluyvera citrophila* [15], 4.75 phenoxymethylpenicillin for the penicillin acylase of *Fusarium semitectum* [18], and 35 mM phenoxymethylpenicillin for the enzyme of *E. aroideae* [9].

For the *B. plumbea* enzyme K_m was calculated from experiments carried out

TABLE II

SUBSTRATE SPECIFICITY OF *B. PLUMBEA* PENICILLIN ACYLASE

Experiments were carried out as described under Materials and Methods.

Substrate	Specific activity	Relative activity (%)
Phenoxymethylpenicillin	26.4	100
p-Hydroxyphenoxymethylpenicillin	26.0	98.5
o-Kresoxymethylpenicillin	5.3	20.1
Benzylpenicillin	1.5	5.7
Thymoxymethylpenicillin	traces	traces
Ampicillin	0	0
Hetacillin *	0	0

* 6-(2,2-dimethyl-5-oxo-4-phenylimidazolidine-2-yl)-penicillanic acid.

with an enzyme solution with a protein concentration of 0.16 mg/ml. The apparent K_m for phenoxymethylpenicillin which was the best substrate for the enzyme was 1.67 mM. No inhibition was observed at concentrations of phenoxymethylpenicillin as high as 51.2 mM. Activation energy was evaluated by Arrhenius' graphical method in a temperature range of 12—62°C. The value obtained was 19.45 kJ/mol. Activation energy for cleavage of phenoxymethylpenicillin by *F. semitectum* enzyme was reported to be 38.5 kJ/mol and 43.9 kJ/mol for benzylpenicillin by the *Escherichia coli* enzyme [25]. Maximum activity of the enzyme was observed at 52°C; at this temperature 1 ml of enzyme protein split 37.1 μmol phenoxymethylpenicillin per min at the optimal pH (7.5) showing no decrease in activity within 10 min.

Inhibition studies

Penicillin acylase of *F. semitectum* was strongly inhibited by metal-chelating agents due to its content of two atoms of zinc per molecule [16].

Differing results were obtained with the enzyme described in this paper: Neither EDTA (over a range from 60 μM to 10 mM) nor 8-hydroxyquinoline (over a range from 60 μM to 26 mM) and iodoacetic acid up to 28 mM showed any characteristic effect. No inhibition occurred with both 50 mM phenoxyacetic acid and 50 mM 6-aminopenicillanic acid, the cleavage products of phenoxymethylpenicillin, as effectors. Thus neither metal atoms nor sulfhydryl groups appear to be essential for enzyme action.

Discussion

A large number of microorganisms have been found to produce penicillin acylase [2—6], but detailed studies on the enzyme have been carried out with only a few species.

The competition within the fermentation industries concerning the strains, their properties and applications prevents publication of the essential data. Most of the published results have been obtained with suspensions of intact cells, crude extracts or slightly purified enzyme preparations. Only recently, data about highly purified penicillin acylases have been published [9,14—19].

Claridge et al. [26] proposed a classification of the enzyme based on the

type of microorganism producing the enzyme, but due to the increasing number of exceptions reported [7—9,11,24,27] the type of microorganism involved seems to be no longer a valid basis for a classification. Summarizing all data published it is justified to classify the penicillin acylases according to the type of penicillin preferably hydrolyzed. Studies on the substrate specificity of the enzyme led to the conclusion that the classical "bacterial" acylases act not only on penicillins but also on amides (e.g. phenylacetamide) and *N*-acylamino acids. *E. coli* enzyme was crystallized and the suggestion was confirmed that penicillins are not the only substrates for penicillin G acylases [19]. On the other hand, there exist penicillin acylases deacylating phenoxymethylpenicillin much faster than other penicillins and lacking specificity towards *N*-acylated amino acids or amides; e.g. in *F. semitectum* a specific phenoxymethylpenicillin acylase was separated from an enzyme activity present in the mycelium deacylating a number of *N*-phenoxyacetylamino acids [18].

A novel type of penicillin acylases, ampicillin acylase, from pseudomonads was described recently [7,8,28]. These enzymes are reported to hydrolyze only ampicillin.

The penicillin acylase from *B. plumbea*, as described here, has a high specificity for phenoxymethylpenicillin and its natural oxidation product, *p*-hydroxyphenoxymethylpenicillin; *o*-kresoxymethylpenicillin and benzylpenicillin are deacylated much slower. No activity is detectable against *N*-acylamino acids and peptides. The molecular weight of 88 000 is different from the values obtained for other purified penicillin acylases [9,14—17,19].

The results obtained justify the designation of the penicillin acylase from *B. plumbea* NRRL 3501 as a new species of phenoxymethylpenicillin acylase.

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References

1 Doyle, F.P. and Nayler, J.H.C. (1965) Adv. Drug Res. 1, 1—69
2 Hamilton-Miller, J.M.T. (1966) Bacteriol. Rev. 30, 761—771
3 Huber, F.M., Chauvette, R.R. and Jackson, B.G. (1972) Cephalosporins and Penicillins (Flynn, E.H., ed.), Academic Press Inc., New York
4 Vandamme, E.J. and Voets, J.P. (1974) Adv. Appl. Microbiol. 17, 311—369
5 Savidge, T.A. and Cole, M. (1975) Methods in Enzymology (Colowick, S.P. and Kaplan, N. O., eds.), Vol. 43, pp. 705—721
6 Vanderhaeghe, H. (1975) Methods in Enzymology (Colowick, S.P. and Kaplan, N.O., eds.), Vol. 43, pp. 721—728
7 Okachi, R., Kato, F., Miyamura, Y. and Nara, T. (1973) Agric. Biol. Chem. 37, 1953—1961
8 Okachi, R. and Nara, T. (1973) Agric. Biol. Chem. 37, 2797—2804
9 Vandamme, E.J. and Voets, J.P. (1975) Experientia 31, 140—143
10 Cole, M. (1964) Nature 203, 519—520
11 Nara, T., Misawa, M., Okachi, R. and Yamamoto, M. (1971) Agric. Biol. Chem. 35, 1676—1684
12 Brandl, E. and Kleiber, W. (1963) Österr. Pat. No. 297 923
13 Brandl, E., Kleiber, W. and Knauseder, F. (1972) Österr. Pat. No. 233 738
14 Chiang, C. and Bennett, R.E. (1967) J. Bacteriol. 93, 302—306
15 Shimizu, M., Okachi, R., Kimura, K. and Nara, T. (1975) Agric. Biol. Chem. 39, 1655—1661
16 Waldschmidt-Leitz, E. and Bretzel, G. (1964) Z. Physiol. Chem. 337, 222—228

17 Takahashi, T., Yamazaki, Y. and Kato, K. (1973) Abstract of Papers, Annual Meeting of Agricultural Chemical Society of Japan, Tokyo, p. 287
18 Baumann, F., Brunner, R. and Roehr, M. (1971) Z. Physiol. Chem. 352, 853—858
19 A.G. Bayer (1972) Japan Patent 47-101 679
20 Edsall, Z. (1973) J. Am. Chem. Soc. 59, 2245—2248
21 Atkinson, A., Bradford, P.A. and Selmes, I.P. (1973) J. Appl. Chem. Biotechnol. 23, 517—529
22 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
23 Warburg, O. and Christian, W. (1941) Biochem. Z. 310, 84—92
24 Roehr, M., Baumann, F. and Brunner, R. (1969) Monatsh. Chem. 100, 236—241
25 Brandl, E. (1965) Z. Physiol. Chem. 342, 86—92
26 Claridge, C.A., Luttinger, J.R. and Lein, J. (1963) Proc. Soc. Exp. Biol. Med. 113, 1008—1012
27 Rossi, D., Romeo, A., Lucente, G. and Tinti, O. (1973) Farmaco, Edn. Sci. 28, 262
28 Nara, T., Okachi, R. and Kato, F. (1972) Abstracts of the 4th International Fermentation Symposium p. 207