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PROPERTIES OF A PARTIALLY - PURIFIED ESTERASE FROM *ASPERGILLUS NIGER* NRRL 337

MOLSIRI VEEROTHAI

Department of Home Economics, Faculty of Science, Srinakarinwirot University (Prasanmitr), Bangkok 10110, Thailand.

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Abstract

An esterase has been isolated from an aqueous extract of a wheat bran culture of Aspergillus niger *NRRL 337 by ammonium sulfate precipitation, followed by chromatography on DEAE Sephadex A - 50, Sephadex G - 100, DEAE Sepharose and Sephadex G - 75. The partially purified enzyme had a specific activity (methyl acetyl salicylate, MAS, as a substrate) of 30.4 units per mg of protein. The apparent K_m for MAS was 7.5 mM. The molecular weight was estimated as about 100,000 by gel filtration and as 120,000 by HPLC. The enzyme catalyzed the preferential hydrolysis of esters derived from short chain fatty acids. A serine residue may participate in the catalysis of hydrolysis by the enzyme. Other molecular characteristics of the enzyme are described.*

Introduction

Lipolytic enzymes, such as lipases and esterases, constitute an important group of enzymes because of their association with fat metabolism as well as with fat degradation. They are widely distributed in both animals and plant, and are physiologically important since they catalyze hydrolysis of fats and oils, giving rise to free fatty acids and partial glycerides, which undergo metabolic processes, including transport, oxidation and resynthesis of glycerides and phospholipids. Consequently, these enzymes are of considerable economic significance in the food industry.

Esterases are enzymes that catalyze the hydrolysis of esters of fatty acids as well as the simple esters of alcohols and aromatic acids. The major difference between lipases and esterases seems to be associated with the state of the substrate they act upon. Whereas an esterase can only catalyze hydrolysis of soluble or fully dispersed substrates, a lipase can catalyze hydrolysis of the esters at an oil-water interface, in a heterogeneous system.

Previously, animals and plants were the main sources of the enzymes, but nowadays these sources are limited. The study of microorganisms which produce these enzymes will help to enhance their production and satisfy the commercial demand for these products. It has been found previously that *Aspergillus niger* produces many

such enzymes[1,2] and this paper describes the partial purification and some properties of an esterase isolated from a strain of *Aspergillus niger* NRRL 337.

Materials and Methods

Microorganism, medium and cultivation.

The organism used in this study was a strain of *Aspergillus niger* NRRL 337 stocked in the Osaka Municipal Technical Research Institute laboratory. The stock culture was first grown in a seed cultivation medium, consisting of 5.3 g of sterile wheat bran, 3.9 g of water and 0.8 g of calcium carbonate, kept at 27° C for 5 days. This seed culture was used to inoculate a wheat bran medium, consisting of 5 kg of wheat bran, 3.75 l of water and 750 g of calcium carbonate. Cultivation was carried out in 10 covered aluminium trays (35 x 55 x 5 cm) at 27° C for 4 days.

Enzyme extraction and purification [3,4,5].

Five parts of water were added to one part of culture and, after being kept for 3 h at room temperature, the mixture was centrifuged at full speed for 20 min.

Solid ammonium sulfate was added to the crude aqueous extract so as to obtain 0.2 saturation. After being kept overnight at 4° C, the solution was filtered through Hyflo Super - Cel previously washed with 0.2 saturated ammonium sulfate solution. Solid ammonium sulfate was then added to the filtrate so as to give 0.75 saturation. After being kept overnight at 4° C, the mixture was filtered through Hyflo Super - Cel previously washed with 0.75 saturated ammonium sulfate solution to collect the precipitate The precipitate was then redissolved in water and the solution was applied to a column of Sephadex G - 75 (10 x 100 cm), previously equilibrated with 0.05 M acetate buffer (pH 5.6), to remove ammonium sulfate.

The eluate was applied to a column of DEAE Sephadex A - 50 (5 x 55 cm) previously equilibrated with 5 mM sodium phosphate buffer (pH 6.0). The enzyme was eluted by a gradient of NaCl formed by controlled mixing of two 1000 ml buffers, with and without 0.5 M NaCl. The flow rate was 50 ml per h. The eluate was collected in fractions of 16 ml and the active fractions (60 - 72) were combined and concentrated by cellulose acetate membrane ultrafiltration.

The enzyme solution was applied to a column of Sephadex G - 100 (4 x 125 cm) conditioned with 5 mM sodium phosphate buffer (pH 6.0). Elution with the same buffer at a flow rate of 52 ml per h gave a series of 15 ml fractions; the active fractions (41 - 60) were again combined and concentrated by cellulose acetate membrane ultrafiltration.

This enzyme solution was applied to a column of DEAE Sepharose (3 x 45 cm) previously equilibrated with 5 mM sodium phosphate buffer (pH6.0). The enzyme was

eluted by a gradient of NaCl formed by controlled mixing of two 600 ml buffers, with and without 0.5 M NaCl. The flow rate was 17 ml per h. The eluate was collected in 8.5 ml fractions and the active fractions (89 - 101) were combined and concentrated by cellulose acetate membrane ultrafiltration as before.

The concentrated enzyme solution was finally applied to a column of Sephadex G - 75 (25 x 100 cm) previously equilibrated with 5 mM sodium phosphate buffer (pH 6.0) Elution was carried out with the same buffer at a flow rate of 7 ml per h. The eluate was collected in 3.8 ml fractions and the active fractions (50 - 66) were again combined and concentrated by cellulose acetate membrane ultrafiltration.

Enzyme assay

The activity of the enzyme was determined with methyl acetyl salicylate (MAS) as substrate. Reaction mixtures (5.0 ml) containing 3.5 ml of 0.01 M MAS, 0.5 ml of 0.5 M sodium phosphate buffer (pH7.0)and 1 ml of enzyme solution were incubated at 35° C for 20 min. The absorbance due to the methyl salicylate (MS) produced was measured at 300 nm. The extinction coefficient $\triangle \varepsilon$ at 300 nm was 0.37.

One unit of enzyme was defined as that amount catalysing the formation of one micro mol equivalent of methyl salicylate per minute under the assay condition.

Determination of protein

The protein was estimated spectrophotometrically by using milk casein as a standard. The specific activity of the enzyme was expressed as enzyme units per O.D. 1.0 at 280 nm.

Preparative disc gel electrophoresis

One millilitre of the concentrated enzyme solution from Sephadex G-75 column was applied on a PD-2/150 column (Canalco Co.) on 7.5% polyacrylamide gel with pH 8.3 buffer system. The thickness of seperating gel was 4 cm. The gel was run at 900 V x 30 mA at 4° C. The protein was eluted with 5 mM phosphate buffer pH 7. One millilitre of the eluate was collected per fraction. The active fraction (fraction No. 18-23) were collected and concentrated by cellulose acetate membrane ultrafiltration.

Analytical disc gel electrophoresis

Analytical disc gel electrophoresis was carried out according to the method of Davis[6] with an apparatus of M.S. Instrument Co. The columns of 7.5% polyacrylamide gel (0.5 x 6 cm) with pH 8.3 buffer system containing 0.1 M β- mercaptoethanol were run at a current of 5 mA per column for 30 min at room temperature. The gels were stained with Amido Black 10 B, and destained with 7% acetic acid.

Molecular weight determination

The molecular weight of the enzyme was determined by HPLC (Hitachi) on a G 3000 SW column 7.5 mmID x 60 cm x 2. The flow rate was 1 ml/min. Phosphate buffer M/15 pH 7 was used for conditioning and elution. Standard proteins used were BSA (MW 67,000) ovalbumin (MW 43,000), chymotrypsinogen A (MW 25,000) and ribonuclease A (MW 13,000).

The molecular weight of the enzyme was also determined by gel filtration on a column of Sephadex G - 100 (1 x 100 cm). Elution was carried out with 5 mM sodium phosphate buffer (pH 7.0). A calibration curve was made with the standard proteins BSA, ovalbumin, chymotrypsinogen A and ribonuclease A, and V_o was determined by blue dextran (MW 2,000,000).

Examination of substrate specificity[7]

A mixture consisting of 0.5 g of substrate, 5 ml of M/50 phosphate buffer (pH 7.0) and 20 µl of enzyme was stirred at 500 rev/min at 50° C. After incubation for 1 h, 20 ml of ethanol was added to the mixture and the liberated fatty acids were determined by titration with 0.05 M KOH.

Effect of diisopropylfluorophosphate (DFP)

It has been reported that most of esterases from animal tissues have a serine residue in their active sites[3]. To determine if a serine residue participates in the catalysis in the present case, DFP was used. Ten microlitres of enzyme were incubated with 400 microlitres of 1 mM of DFP at 30° C for 20 min. The enzyme activity of the test sample to which DFP was added was compared with the control sample[8] (MAS as a substrate)

Results and Discussions

Fig 1 shows an elution profile of the esterase obtained from a strain of *Aspergillus niger* NRRL 337, from a DEAE Sephadex A - 50 column. An elution profile of the enzyme from a Sephadex G - 100 column is shown in Fig 2. Figs 3 and 4 show elution profiles of the enzyme from DEAE Sepharose and Sephadex G - 75 columns, respectively. Judging from the profile in Fig 4 the enzyme is not yet completely purified.

The results of experiments to purify the enzyme are summarized in Table 1. These show that about 1,150 fold purification at a yield of 4% has been achieved. The enzyme had a specific activity (MAS as substrate) of 30.4 units per mg of protein. Some properties of the enzyme are summarized in Table 2.

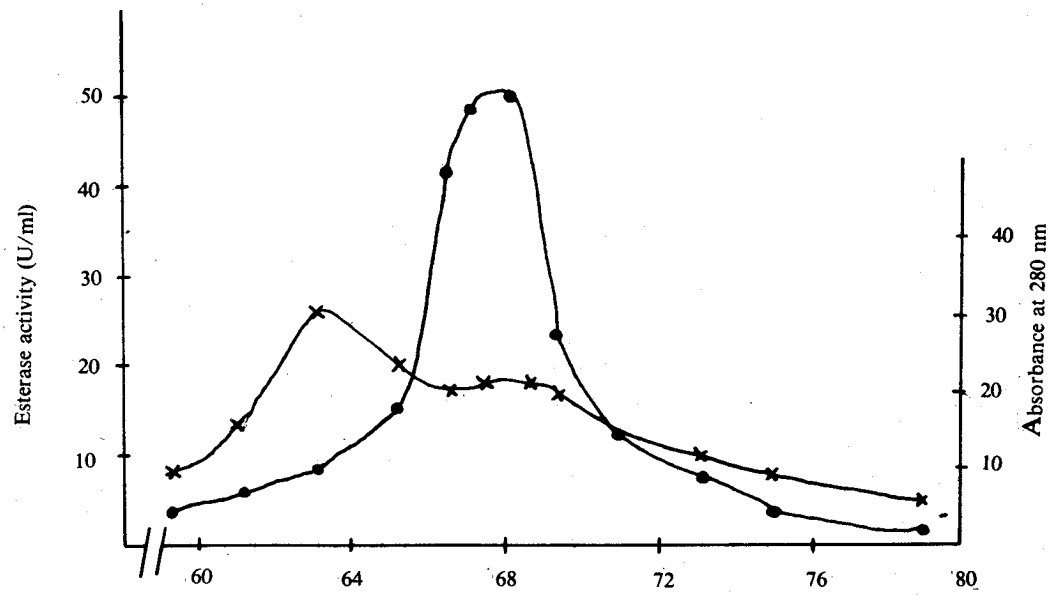

Fig 1. Column Chromatography of the Esterase on DEAE Sephadex A - 50

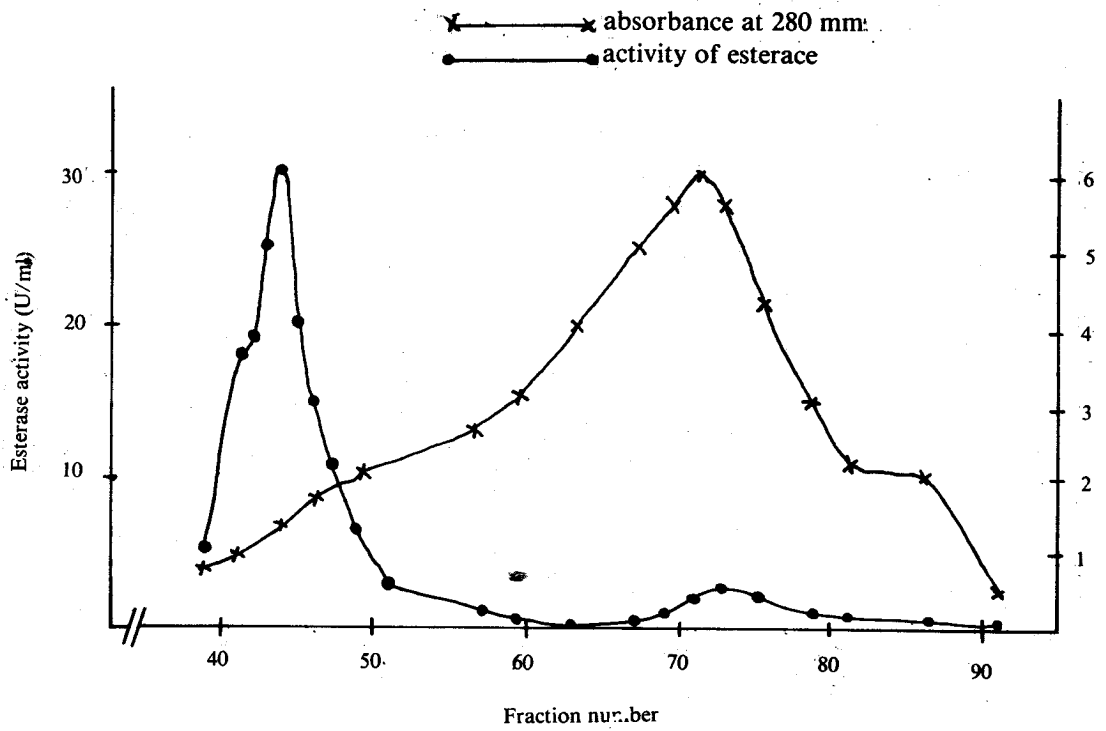

Fig 2. Sephadex G - 100 Gel Filtration.

absorbance at 280 nm
activity of esterase

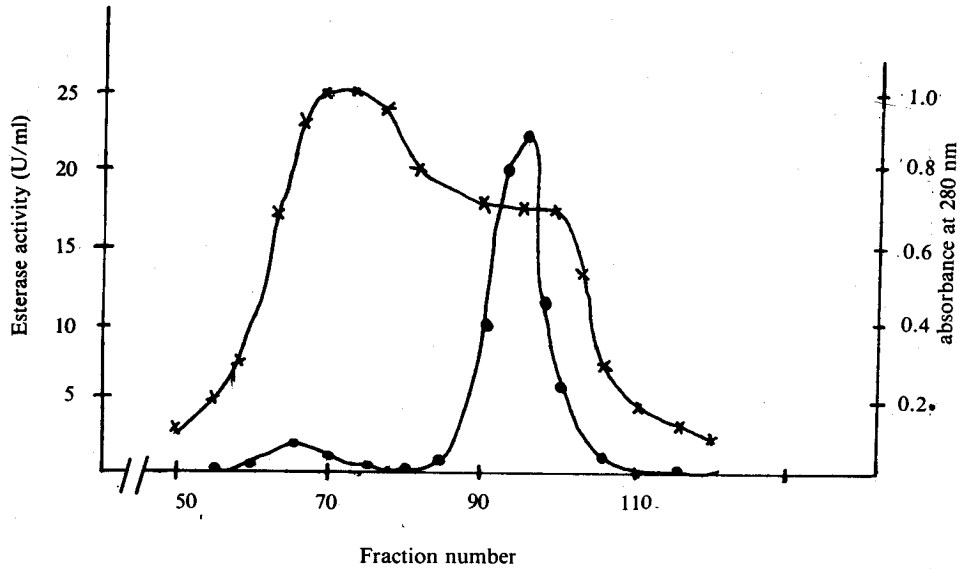

Fig 3. DEAE Sepharose Column Chromatography

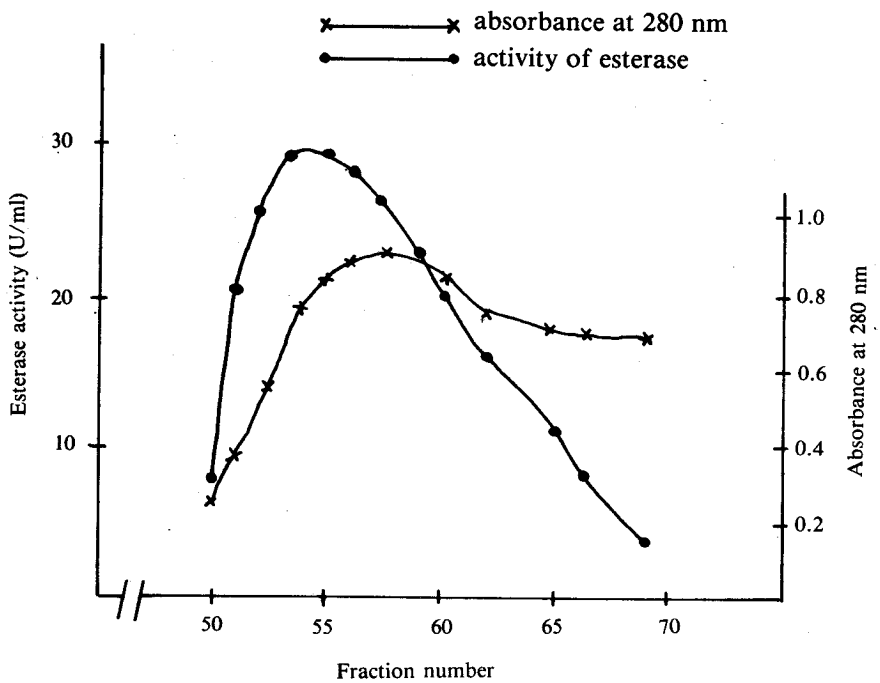

Fig 4. Sephadex G - 75 Chromatography

absorbance at 280 nm
activity of esterase

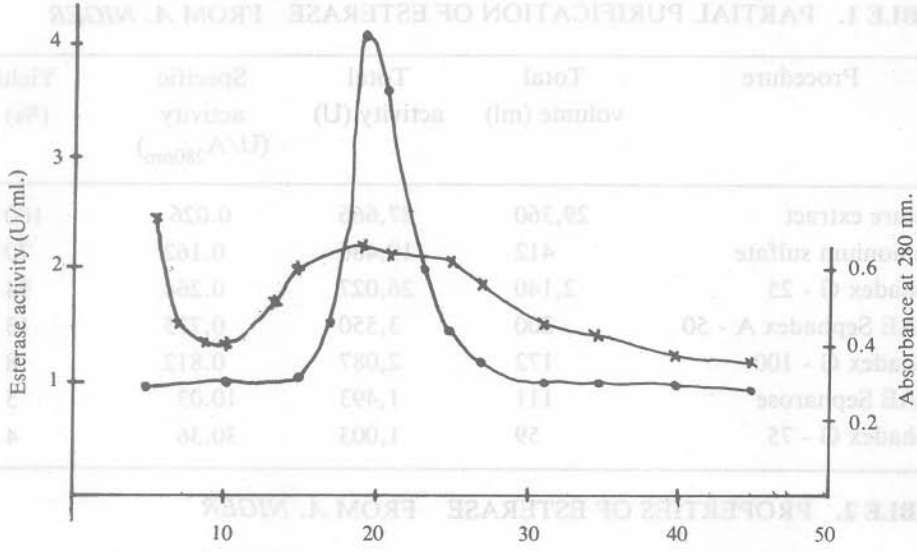

Fig 5. Preparative Disc Gel Electrophoresis Chromatography
The column of 7.5% polyacrylamide disc gel was run at 900 volt for 1 h at 4° C and 1 ml of efluent was collected per fraction.
●——● absorbance at 280 nm.
×——× activity of esterase

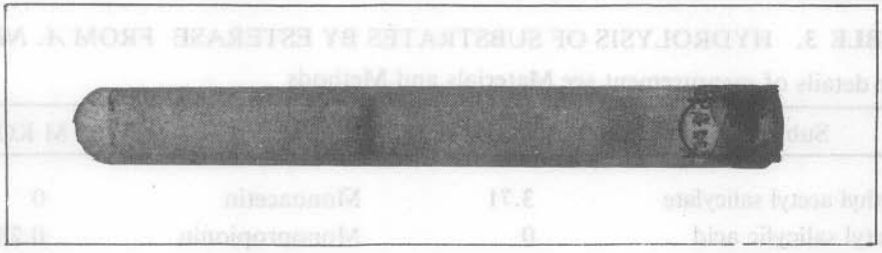

Fig 6. Disc Gel Electrophoresis of Partially Purified Esterase

TABLE 1. PARTIAL PURIFICATION OF ESTERASE FROM *A. NIGER*

Procedure	Total volume (ml)	Total activity (U)	Specific activity (U/A_{280nm})	Yield (%)
Culture extract	29,360	27,666	0.026	100
Ammonium sulfate	412	19,486	0.162	70
Sephadex G - 25	2,140	26,027	0.264	94
DEAE Sephadex A - 50	200	3,550	0.775	13
Sephadex G - 100	172	2,087	0.812	8
DEAE Sepharose	111	1,493	10.03	5
Sephadex G - 75	59	1,003	30.36	4

TABLE 2. PROPERTIES OF ESTERASE FROM *A. NIGER*

Molecular weight	100,000 (by gel filtration) 120,000 (by HPLC)
Optimum pH (35° C, 20 min)	7
pH stability (30° C, 16 h)	6 - 8
Optimum temperature (pH 7,20 min)	60° C
Thermal stability (pH 7,15 min)	$< 70^{\circ}$ C
K_m for MAS	7.5 mM

TABLE 3. HYDROLYSIS OF SUBSTRATES BY ESTERASE FROM *A. NIGER*
For details of measurement see Materials and Methods

Substrate	0.05 M KOH (ml)	Substrate	0.05 M KOH (ml)
Methyl acetyl salicylate	3.71	Monoacetin	0
Acetyl salicylic acid	0	Monopropionin	0.28
Phenyl acetate	1.88	Monoolein	0.30
Methyl salicylate	0		
Benzyl acetate	0	Triacetin	0
Phenylethyl acetate	0	Tripropionin	1.15
		Tributyrin	1.62
Methyl acetate	0	Tricaproin	0.98
Methyl propionate	0	Tricaprylin	0
Methyl butyrate	0	Tricaprin	0
Methyl caproate	0.27	Triolein	0
Methyl caprylate	1.48		
Methyl caprate	0.45		
Methyl oleate	0.20	Ethyl gallate	0

The molecular weight of the enzyme was determined to be about 120,000 by gel filtration on Sephadex G - 100 and about 100,000 by HPLC. The effects of pH and temperature on the enzyme activity were examined and the results are summarized in Table 2.

Table 3 gives information on the relative activity for various substrates. Among the aromatic esters, methyl esters, mono - and triglycerides, and polyphenolic compounds tested as substrates, the enzyme hydrolyzed methyl acetyl salicylate with the highest velocity; the enzyme also preferentially hydrolyzed triglycerides derived from short chain fatty acids.

The partially purified enzyme migrated as a single protein band and had an activity band with the same mobility as the protein band in dise electrophoresis (Fig 6) The enzyme was 86% inactivated after incubation with DFP at 30° C for 20 min. This suggests that serine residue participates in catalysis by the enzyme.

Acknowledgements

I wish to express my hearty thanks to Dr. Susumu Okumura, Dr. Mieko Iwai and Dr. Yoshio Tominaka from the Osaka Municipal Technical Research Institute for valuable advice and encouragement.

References

1. Fukumoto, J., Iwai, M., and Tsujisaka, Y. (1964) *J. Gen. Appl. Microbiol.* **10**, 257.
2. Iwai, M., Tsujisaka, Y., and Fukumoto, J. (1970). *J. Gen. Appl. Microbiol.* **16**, 181.
3. Krisch, K. (1971) *The Enzymes.* Boyer, P.D., ed., vol 5, pp 43-69, Academic Press, New York.
4. Tsujisaka, Y, Iwai, M., and Tominaka, Y. (1973). *Agr. Biol. Chem.* **37**, 1457.
5. Iwai, M., and Tsujisaka, Y. (1974). *Agr. Biol. Chem.* **38**, 1241.
6. Davis, B.J. (1964). *Ann. N.Y. Acad. Sci.* **121**, 404.
7. Okumura, S,Iwai, M, and Tsujisaka, Y. (1980). *J. Biochem.* **87**, 205.
8. Krisch, K. (1970). *Z. Klin. Chem. Klin. Biochem.* **8**, 545.

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2. Iwai, M., Tsujisaka, Y., and Fukumoto, J. (1970). *J. Gen. Appl. Microbiol.* **16,** 181.
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7. Okumura, S,Iwai, M, and Tsujisaka, Y. (1980). *J. Biochem.* **87,** 205.
8. Krisch, K. (1970). *Z. Klin. Chem. Klin. Biochem.* **8,** 545.