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# SH-PROTEINASE FROM BEAN *PHASEOLUS VULGARIS* VAR. *PERLICKA*

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## Summary

An SH-proteinase (EC 3.4.22.—) has been isolated from beans of the species *Phaseolus vulgaris* var. *Perlička*. The enzyme is homogeneous when subjected to disc electrophoresis, electrofocusing and sedimentation analysis. The molecular weight was determined as 26 000—28 000 by gel filtration, 30 850 ± 1500 by sedimentation analysis and 26 930—27 410 by calculation from the amino acid composition ($Lys_{20-21}$, $His_3$, $Arg_9$, $Asp_{21-22}$, $Thr_{13}$, $Ser_{18}$, $Pro_{12-13}$, $Glu_{23-24}$, $Gly_{30}$, $Ala_{16}$, $Cys/2_9$, $Val_{19}$, $Met_1$, $Ile_{10}$, $Leu_{13}$, $Tyr_{14}$, $Phe_6$, $Trp_3$). The N-terminal amino acid of the proteinase is isoleucine. The effect of concentration, time of hydrolysis, pH, temperature, cations, anions, urea and guanidine · HCl on the proteolytic activity of the SH-proteinase was studied.

## Introduction

Little is known about proteolytic enzymes isolated from leguminous plants even though it appears from the data obtained during the past few years that various types of bean may represent a rich source of proteinases.

The proteinase from soy bean is one of the first proteinases ever isolated from leguminous plants; it is obviously an SH-proteinase, with optimum activity at pH 5.5 [1,2]. A proteinase of the same type has also been isolated from Lima beans [3].

A mixture of proteolytic enzymes can be extracted from the kidney-shaped bean, *Phaseolus vulgaris*, which shows activity of the chymotrypsin, leucine aminopeptidase and trypsin type [4]. A so-far unspecified proteinase has been isolated from the seedlings of the bean *Phaseolus aureus Rexb* [5].

An endopeptidase has been isolated from the bean *Phaseolus angularis*. Inhibition studies have shown that endopeptidase is not an SH-proteinase, but its type has not been determined so far [6]. The leaves of the bean *Phaseolus*

*vulgaris* var. *pencil* contain an enzyme which hydrolyzes casein and hemoglobin to long peptide fragments [7]. The type of this proteinase has not been defined as yet, but it is assumed that it strongly resembles the proteinase which accompanies phaseolain. Phaseolain is a carboxypeptidase first isolated by Wells [8] together with other proteolytic enzymes (so far designated proteinases a, b and c) from French beans. Of this population of enzymes, phaseolain has been studied most and is the most interesting one because of its unique structure around the active site [9—11]. It has been postulated that one of the proteinases mentioned is of the SH-type. The SH-proteinase isolated by us will be compared with papain, ficin and bromelain, which are the plant SH-proteinases most studied so far.

## Materials and Methods

The SH-proteinase was isolated from the bean *Phaseolus vulgaris* var. *Perlička* by covalent chromatography on mercury derivatives of hydroxyalkylmethacrylate gels [12] and purified by chromatography on a column of Sephadex G-75 (2.5 × 43 cm), equilibrated in 0.02 M ammonium acetate, pH 6 (Fig. 1). The proteolytic activity was determined by a modification [13] of the method of Anson [14]. The number of free SH groups was determined according to Robyt et al. [15].

*Homogeneity of SH-proteinase*

Disc electrophoresis in 7.5% polyacrylamide gel and β-alanine buffer, pH 4.3, was carried out according to Nagai et al. [16]. The mobility of 0.5 and 1.0% solutions of the SH-proteinase was examined by electrophoresis runs of 1, 2 and 3 h.

The sedimentation coefficient of 0.25, 0.5, 0.75 and 1% solutions of the SH-proteinase in 0.05 M $NH_4HCO_3$ was measured in Spinco Model E ultracentrifuge at 59 780 rev./min (Fig. 2).

The isoelectric point was established by electrofocusing in a sucrose gradient at pH 3—10 in LKB 8101 apparatus, by the procedure recommended in the LKB manual [18]. The focusing was allowed to proceed for 44 h at 300 V (Fig. 3).

*Characterization of proteinase*

The molecular weight was determined in the ultracentrifuge by the method of Yphantis [19] and by gel filtration on a column of Sephadex G-150 (2.5 × 48 cm) equilibrated in 0.02 M ammonium acetate at pH 6 [20,21] (Fig. 4).

The quantitative amino acid analysis was performed by the method of Spackman et al. [22] and the results are given in Table I. The values shown in the Table are means of three determinations carried out with both oxidized and unoxidized samples.

The sugar content was determined qualitatively by the anthrone method [23].

Dependence of proteolytic activity on time of hydrolysis was investigated. A proteinase solution (0.5 mg/100 μl) was pipetted into test-tubes containing the hemoglobin solution at pH 5, 8 and 10. Unhydrolyzed hemoglobin was

precipitated by 5% trichloroacetic acid after 5, 10, 20, 30, 40, 50, 60, 70, 80 and 90 min of hydrolysis (Fig. 5).

*Investigation of stability of SH-proteinase*

The stability of SH-proteinase as function of pH was investigated by adding a proteinase solution (2 mg/0.1 ml) to 2 ml of Britton-Robinson [24] buffer at various pH values. The mixture was allowed to stand 10 min, 3 and 24 h at room temperature. After this period, 0.2 ml of the activating solution (0.05 N Cys/0.02 M EDTA, pH 8.5) was added and the proteolytic activity was determined at pH 8 (Fig. 6).

To investigate the effect of temperature on proteolytic activity, the proteolytic activity of the proteinase was determined after 0.5 h incubation of a 0.05% solution of the SH-proteinase in water (pH 5.8), 0.005 M $CaCl_2$ (pH 6.2) and 0.01 M NaCl (pH 6.1) at temperature values of 4, 20, 30, 40, 50, 60, 70, 80 and 90° C. The activity was determined after activation of the proteinase samples immediately before the assay (Fig. 7).

To determine the effect of cations on proteolytic activity, a 0.05% solution of the SH-proteinase in 0.1 M Tris · HCl buffer at pH 7 was incubated in the presence of 0.1 and 0.001 M solutions of various cations for 10 min at 37° C. The activity was determined after 10 min of incubation, and is expressed in relative per cent, taking the activity of 0.05% solution of the proteinase in 0.01 M Tris · HCl buffer, pH 7, after incubation in the absence of metal ions to represent 100%.

To determine the effect of anions on proteolytic activity, 0.05% solutions of the proteinase in 0.1 M solutions of sodium salts were prepared and allowed to stand 10 min, 0.5, 1 and 3 h at room temperature. Samples of the solutions were removed and their proteolytic activity determined. The activity of a 0.05% solution of the proteinase in water was taken to represent 100%.

The effect of urea and guanidine · HCl on proteolytic activity was investigated by determining the activity of a 0.05% solution of the SH-proteinase incubated 0.5 h at room temperature in solutions of urea and guanidine · HCl of various molarity (Fig. 8).

Comparison of the inactivation of SH-proteinase, papain and ficin was made by determining the residual proteolytic activity of a 0.1% solution of the SH-proteinase, papain or ficin in water and 0.1 M sodium acetate at pH 5 and 37° C, as a function of time.

## Results and Discussion

The SH-proteinase isolated from the bean *Phaseolus vulgaris* var. *Perlička* by chromatography on Hg derivatives of hydroxyalkylmethacrylate gels [12] was separated into two fractions by chromatography on Sephadex G-75, as shown in Fig. 1. The fractions in the second peak were lacking in proteolytic activity. The proteolytic activity of the material before the purification procedure is 0.120 units $A_{280}$/min per mg, and of the pure SH-proteinase (first peak) is 0.147 units $A_{280}$/min per mg. The purified enzyme has one free sulfhydryl group and four disulfide groups. The material contained in the first peak was found to be a homogeneous protein when subjected to ultracentri-

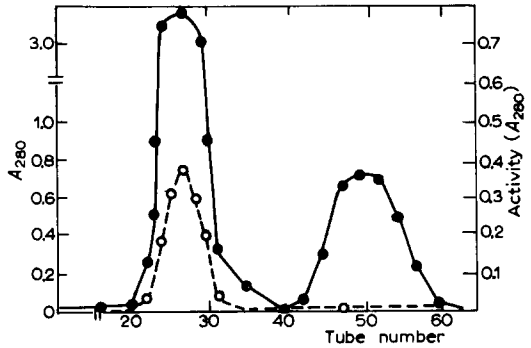


Fig. 1. Purification of SH-proteinase on a column of Sephadex G-75 (2.5 × 43 cm) equilibrated with 0.02 M ammonium acetate, pH 6. The same buffer was used as eluent, and 2-ml fractions were collected at 15-min intervals, 0.2 g was chromatographed. (●), Absorbance at 280 nm; (○), proteolytic activity at pH 8.

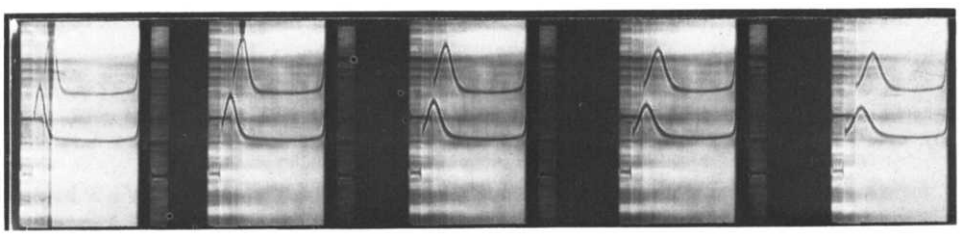

Fig. 2. Sedimentation pattern of SH-proteinase. 0.75% and 1% solutions of the enzyme in 0.2 M $NH_4HCO_3$ were used. The photographs were taken at 59 780 rev./min in Spinco model E ultracentrifuge after 30, 60, 78, 94 and 110 min.

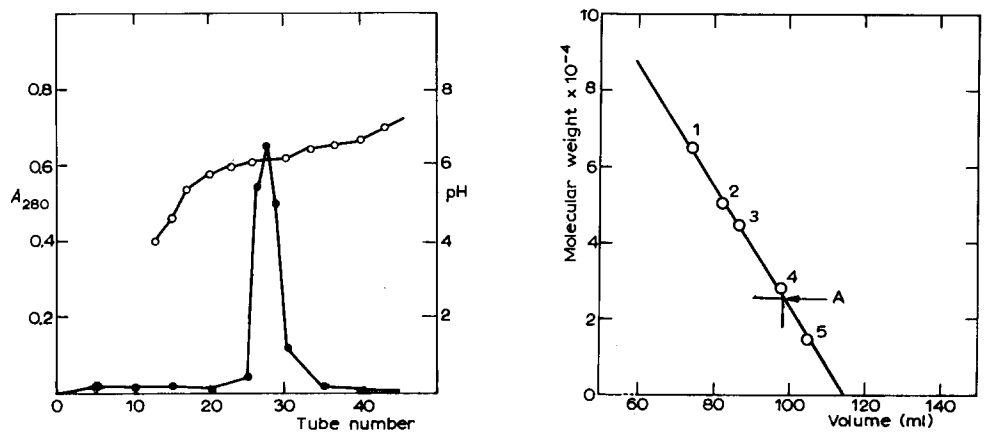


Fig. 3. Determination of p*I* by electrofocusing. The experiment was carried out in a 110-ml column with wide-range ampholine, pH 3—10. The focusing was allowed to proceed for 44 h at 300 V, 5 mg of the sample was applied to the column, and 1.5 ml fractions were collected for determinations. (●), $A_{280}$; (○), pH.

Fig. 4. Determination of molecular weight by gel filtration on a column of Sephadex G-150 (2.5 × 48 cm) in 0.02 M ammonium acetate, pH 6, at 5° C; 3.7-ml fractions were collected at 15-min intervals. Standards used in the experiment: 1, ribonuclease [35]; 2, subtilisin [36]; 3, ovalbumin [37]; 4, pig pancreatic amylase [38]; 5, human serum albumin [19]. A is our preparation of SH-proteinase.

fugation (Fig. 2), disc electrophoresis and electrofocusing (Fig. 3). The protein contained one N-terminal group only, i.e. isoleucine.

The sedimentation coefficient ($S_{20,w}$ = 2.75 S) of the protein is independent of concentration. The isoelectric point determined by electrofocusing is p*I* = 6.34.

The molecular weight determination carried out by the centrifugation method of Yphantis gave a value of 30 850 ± 1500. The molecular weight value found by gel filtration (Fig. 4) varied between 26 000 and 28 000, and the value calculated from the amino acid composition, given in Table I, was 26 930—27 410. For reasons of comparison, the amino acid compositions of papain, ficin and bromelain are also given in Table I. The N-terminal isoleucine found in the SH-proteinase from bean is identical with the N-terminal amino acid of papain. Similar to papain and unlike bromelain and ficin, the SH-proteinase does not contain a sugar moiety.

Table II shows some physicochemical characteristics of the SH-proteinase, together with the corresponding constants for papain, ficin and bromelain.

The dependence of proteolytic activity on the time of hydrolysis is given in Fig. 5.

The SH-proteinase from bean is stable in buffers at pH 6—10 (Fig. 6) and is irreversibly inactivated at higher or lower pH values.

The proteinase is inactivated at temperatures higher than 60° C (Fig. 7) in all the buffers used. Thermal inactivation is effected by the addition of salt.

TABLE I

AMINO ACID COMPOSITION AND N-TERMINAL AMINO ACID OF PAPAIN, FICIN, BROMELAIN AND SH-PROTEINASE FROM BEAN

Number of residues per molecule.

| Amino acid | Papain (34) | Ficin (25) | Bromelain (29) | SH-proteinase |
|---|---|---|---|---|
| Lysine | 10 | 5 | 23 | 20— 21 |
| Histidine | 2 | 1 | 2 | 3 |
| Arginine | 12 | 10 | 12 | 9 |
| Aspartic acid | 19 | 17 | 29 | 21— 22 |
| Glutamic acid | 20 | 25 | 23 | 23— 24 |
| Threonine | 8 | 8 | 14 | 13 |
| Serine | 13 | 14 | 28 | 18 |
| Proline | 10 | 11 | 14 | 12— 13 |
| Glycine | 28 | 28 | 35 | 30 |
| Alanine | 14 | 20 | 35 | 16 |
| Valine | 18 | 18 | 22 | 19 |
| Isoleucine | 12 | 7 | 21 | 10 |
| Leucine | 11 | 15 | 10 | 13 |
| Tyrosine | 19 | 15 | 21 | 14 |
| Phenylalanine | 4 | 5 | 9 | 6 |
| Tryptophan | 5 | 6 | 8 | 3 |
| Half-cysteine | 7 | 8 | 10 | 9 |
| Methionine | 0 | 5 | 5 | 1 |
| Total | 212 | 218 | 321 | 240—244 |
| N-terminal amino acid | Ile [32] | Leu [25] | Val Ala [28] | Ile |

* Bromelain is not homogenous by this criterion.

TABLE II

COMPARISON OF PHYSICOCHEMICAL CONSTANTS OF SH-PROTEINASES FROM PLANTS

| | Papain | Ficin | Bromelain | SH-proteinase |
|---|---|---|---|---|
| Molecular weight by | | | | |
| Molecular sieve | — | — | 22500 ± 1500 | 26000—28000 |
| Ultracentrifugation | 21000 [31] | 25000 ± 750 [25] | 28400 ± 1400 [28] | 30850 ± 1500 |
| Amino acid composition | 23406 [33] | 23800 ± 700 [25] | 25730 [29] | 26430—27412 |
| Isoelectric point | 8.75 [31] | 9.0 [26] | 9.5 [30] | 6.34 |
| $S^{\circ}_{20,w}$ | 2.42 ± 0.045 S [31] | 2.55 S [27] | 2.94 S [28] | 2.75 ± 3% S |

A number of cations and anions affect the proteolytic activity. Mercury cations caused total inactivation, in the presence of $Na^{+}$ and $Ca^{2+}$ the proteinase is slightly activated, and acetates and sulfates activated the proteinase during long periods of incubation. The activating effect of other anions ($Cl^{-}$, phosphate, citrate, tetraborate) is short-term. Fig. 8 shows the effect of urea and guanidine · HCl on the activity of SH-proteinase. It is apparent that both agents cause irreversible inactivation. The influence of the dilution of the denaturing agent, which could result in the reactivation of the enzyme was not pursued. The stability of the SH-proteinase from bean and of papain and ficin in water and in 0.1 M sodium acetate at pH 5 and 37°C is compared; all three proteinases show the same course of inactivation.

Studies aimed at the determination of the degree of similarity in primary structure between the proteinase isolated by us and papain are in progress.

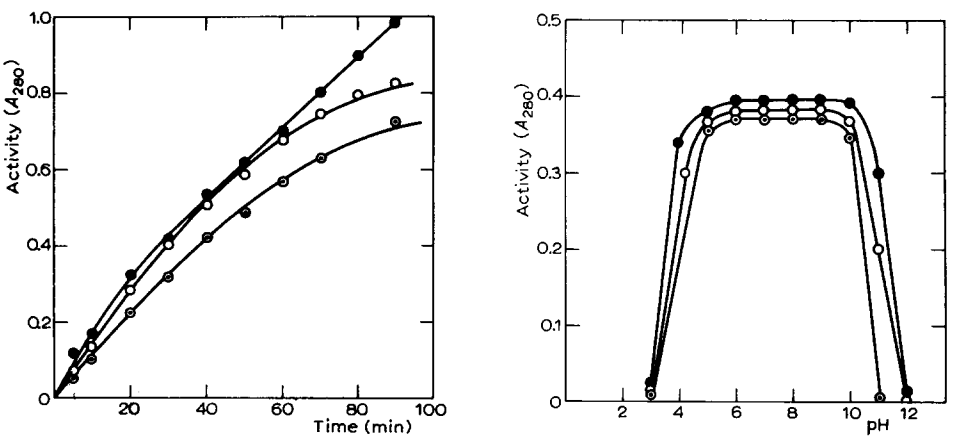


Fig. 5. Effect of time of hydrolysis on proteolytic activity of SH-proteinase. Sample concentration 0.5 mg/100 µl. Substrate: (●), hemoglobin, pH 8; (○), hemoglobin, pH 10; (○), hemoglobin, pH 5.

Fig. 6. Relationship between pH and stability of SH-proteinase. A proteinase solution (2 mg/100 µl) was incubated for (●) 15 min, (○) 3 h, (⊙) 24 h; proteolytic activity was determined with hemoglobin, pH 8, as substrate after the activation of sample with the activating solution (0.05 M cystein/0.2 M EDTA, pH 8.5). $A_{280}$ proteolytic activity according to Anson [14].

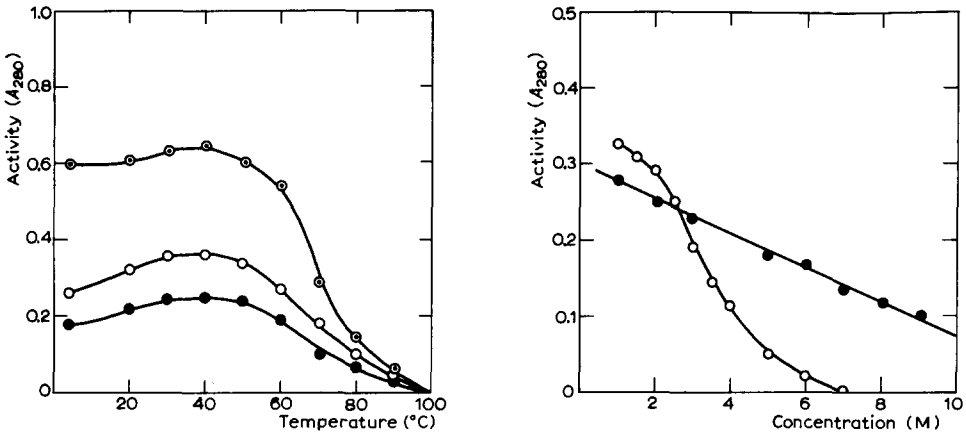


Fig. 7. Relationship between temperature and stability of solutions of SH-proteinase. A 0.05% solution of SH-proteinase was thermostatted 30 min in water (●), in 0.01 M NaCl (○) and in 0.1 M $CaCl_2$ (⊙). The activity was determined after activation of sample at pH 8. $A_{280}$ proteolytic activity according to Anson [14].

Fig. 8. Effect of urea and guanidine · HCl on activity of SH-proteinase. A 0.05% solution of the proteinase after 30 min incubation in solution of urea (●) or guanidine · HCl (○). $A_{280}$ proteolytic activity according to Anson [14].

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## References

1 Weil, J., Pinsky, A. and Gorssman, S. (1966) Cereal Chem. 43, 392—397
2 Tazawa, J. and Hirokawa, T. (1956) J. Biochem. Tokyo 43, 785—795
3 Kudriashova, N.A. (1960) Soobshch. Moskv. Odděla Vshesoi. Botan. Obshch. 83—88
4 Pusztai, A. and Duncan, J. (1971) Planta (Bul.) 96, 317—325
5 Blagoveshschenskij, A.V. (1968) Dokl. Acad. nauk SSSR, 120, 356—361
6 Akune, S. and Takagi, S. (1962) Agr. Biol. Chem. 26, 63
7 Racusen, D. and Foote, M. (1970) Can. J. Bot. 48, 1057—61
8 Wells, J.R.E. (1968) Biochim. Biophys. Acta 167, 388—98
9 Carrey, W.J. and Wells, J.R.E. (1970) Biochem. Biophys. Res. Commun. 41, 574—81
10 Shaw, D.G. and Wells, J.R.E. (1967) Biochem. J. 104, 5C
11 Carrey, W.F. and Wells, J.R.E. (1972) J. Biol. Chem. 247, 5573—79
12 Turková, J., Vavreinová, S., Křiváková, M. and Čoupek, J. (1975) Biochim. Biophys. Acta 386, 503—508
13 Turková, J., Mikeš, O., Gančev, K. and Boublík, M. (1969) Biochim. Biophys. Acta 178, 100—111
14 Anson, M.L. (1939) J. Gen. Physiol. 22, 79—89
15 Robyt, J.F., Ackerman, R.J. and Chittenden, C.G. (1971) Arch. Biochem. Biophys. 147, 262—269
16 Nagai, J., Gross, J. and Piez, K.A. (1964) Ann. N.Y. Acad. Sci. 121, 494—500
17 Gray, W.R. and Smith, J.S. (1970) Anal. Chem. 33, 36—42
18 LKB 81 01 Manual LKB Produkter AB, Stockholm
19 Yphantis, D.A. (1964) Biochemistry 3, 297—317
20 Gelotte, B. (1960) J. Chromatog. 3, 330—42

21 Alcrina, M. and D.C. Battle (1967) J. Chromatog. 28, 82—88
22 Spackmann, D.H., Stein, W.H. and Morre, S. (1958) Anal. Chem. 30, 1190—1206
23 Keil, B., Šormová, Z. (1959) Laboratorni technika biochemie N ČSAV, Prague, p. 501
24 Keil, B., Šormová, Z. (1959) Laboratorni technika biochemie N ČSAV, Prague, p. 747
25 Englund, P.T., King, T.P., Craig, L.C. and Walti, A. (1970) Biochemistry 7, 163—74
26 Cohen, W. (1958) Nature 182, 659—60
27 Metrione, R.M., Johnston, R.B. and Seng, R. (1967) Arch. Biochem. Biophys. 122, 137—43
28 Wharton, C.W. (1974) Biochem. J. 143, 575—86
29 Ota, S., Moore, S. and Stein, W.H. (1964) Biochemistry 3, 180—185
30 Murachi, T., Yasui, M. and Yasuda, J. (1964) Biochemistry 3, 48—55
31 Smit, C.L., Kimmel, J.R. and Brown, D.M. (1954) J. Biol. Chem. 207, 533—49
32 Kimmel, J.R., Thompson, E.O.R. and Smith, E.L. (1955) J. Biol. Chem. 217, 151—9
33 Smith, E.L. and Kimmel, J.R. (1960) in The Enzymes IV, 2nd edn, p. 137, Academic Press, New York
34 Mitchel, R.E.J., Chainken, J.M. and Smith, E.L. (1970) J. Biol. Chem. 245, 3485—90
35 Hirs, C.H.W., Moore, S. and Stein, W.H. (1956) J. Biol. Chem. 219, 623—641
36 Matsubare, H., Kasper, C.B. and Smith, E.L. (1965) J. Biol. Chem. 240, 1125—1130
37 Tristram, G.R. (1949) Adv. Protein Chem. 5, 83—98
38 Caldwall, M.L., Diskey, E.S., Haurahan, V.M., Kung, H.C., Kung, J.T. and Misko, M. (1954) J. Am. Chem. Soc. 76, 143—151