SPECIFICITY AND ESSENTIAL GROUPS IN ALHAGAIN°

K. YESHODHA, S. C. DHAR & M. SANTAPPA
Central Leather Research Institute, Madras.

Received on January 10, 1978.

A systematic investigation has been made to determine the relationship between the proteolytic activity and the reactive groups of alhagain. The effects of certain oxidising, reducing, - SH group blocking agents, metal chelating agents, metallic ions and certain other specific agents on the reactive groups and the proteolytic activity of the enzyme have been studied. The results show that - SH group of the enzyme may be essential for its activity.

The specificity of alhagain has been studied by quantitatively estimating the free amino acids, N - and C - terminal amino acids of the peptides liberated from the hydrolysis of B - chain of oxidised insulin for 6 hours. With reference to the known structure of the B - chain, the major sites of action of alhagain have been determined. Results indicate that alhagain preferentially attacks peptide bonds involving the amino or carboxyl groups of phenyl alanine, amino groups of alanine and serine and also carboxyl groups of lysine and glycine.

Introduction

The activity of an enzyme is generally dependant on the essential reactive groups present in the active centre. In order to determine the essential reactive group of an enzyme, studies on the chemical modifications of the reactive groups and the action of certain specific reagents on the activity of the enzyme are necessary. The sulphydryl group has been observed to be essential for the activity of ficin[1] and calotropain[2]. The phenolic group has been shown to be essential for solanain activity[3]. Phenolic and sulphydryl groups have been found to be essential for the activity of papain[4,5]. The action of certain specific reagents on the activity of various plant proteases viz., papain[6,7], ficin[8], sorghum acid protease[9], alkaline protease from *Agave americana variegata*[10], proteinase from *Phaseolus vulgarisvar Perlicka*[11] and actinidin[12] have been reported in recent years.

Knowledge of the specificity of enzymes is helpful in understanding their mechanism of action. Papain is known to possess amidase and esterase activities and is

° Presented at the 46th Annual meeting of the society of Biological Chemists (India), Madras-September 1977. Forms part of the thesis of one of the authors (KY) approved for the award of Ph. D. degree of the Madras University (1978).

Abbreviations

BAEE, ∝-N-Benzoyl-L arginine ethyl ester; BAME, ∝-N-Benzoyl-L-arginine methyl ester; BAA, ∝-N-Benzoyl-L-arginine amide; BAPNA, ∝-N-Benzoyl-DL-arginine-p-nitroanilide; ATEE, N-Acetyl-L-tyrosine ethyl ester; CBZ, Carbobenzoxy; DFP, Diisopropyl fluoro phosphate; PCMB, p-chloromercuribenzoic acid; EDTA, Ethylene diamine tetra acetate.

capable of hydrolysing those bonds which are formed by the basic amino acids not substituted on the side chain amino group[13]. Phaseolain is reported to have a broad specificity and is found to release acidic, basic and neutral amino acid residues as well as proline, although adjacent acidic residues in a peptide appear to inhibit the enzyme[14]. McDowall[15] has reported the action of proteinase A, of *Actinidia chinensis* on the B-chain of oxidised insulin. The specificity of four sulphydryl endopeptidases ("Ficins") of *Ficus glabrata* latex on the B-chain of oxidised insulin, casein and synthetic ester substrates has been reported[16]. Stem bromelain has been found to possess a relatively broad specificity against B-chain of oxidised insulin and glucagon[17]. A comparative study of specificity of ficin with that of bromelain has been carried out by Kortt *et al*[18] using synthetic substrates, A- and B-chains of insulin. On studying the specificity of the sorghum acid protease with synthetic peptides and ribonuclease A, Garg and Virupaksha[19] have found that the enzyme specifically cleaves the peptide linkages involving the α-Carboxyl group of either aspartic acid or glutamic acid with the release of acyl portion of these amino acids. The enzyme has also been found to have a requirement for unsubstituted side chain carboxyl groups of aspartic and glutamic acids in its substrates. The hydrolysis of peptide bonds formed by the carboxyl group of aromatic amino acids and the branched aliphatic amino acids by agave protease has been reported.[10] The enzyme has not been found to hydrolyse small substrates like dipeptides.

In the present investigation, a systematic study was undertaken in order to find out the nature of the reactive groups of alhagain that are essential for its enzymic activity. Studies on the specificity of action of alhagain on a standard polypeptide chain viz, the B-chain of oxidised insulin and on certain synthetic substrates have also been carried out.

Materials and Methods

Materials

BAEE, BAA, BAPNA, N-CBZ-L-Glutamyl-L-Tyrosine and DFP were purchased from Sigma Chemical Company, St. Louis, U.S.A. BAME and PCMB were obtained from Koch-Light Laboratories Ltd., Colnbrook, England. CBZ-Glycyl-L-Tryptophan and N Acetyl-L-Tyrosine amide were supplied by Mann Research Laboratories, Inc., New York. Crystalline insulin, ATEE and 8-hydroxy quinoline were purchased from BDH Chemicals Ltd., Poole, England. Glutathione, ovomucoid and *O*-phenanthrolin hydrate were obtained from S.A.F., Hoffmann-LA. Roche and Co. Ltd., Switzerland, V.P. Chest Institute, Delhi and E. Merck, W. Germany respectively. All other chemicals were of analytical or reagent grade.

Enzyme used

Alhagain, the neutral protease present in the leaves and barks of jawasee shrub was purified to a homogeneous product according to the procedure described by Yeshodha *et al.*[10] Sufficient amount of alhagain was prepared, stored in the refrigerator and was used throughout the present investigation.

Enzyme assay

The proteolytic activity of alhagain was estimated by colorimetric method as described by Yeshodha *et al.*[11] The proteolytic activity is expressed as unit of enzyme activity and is defined as the amount of enzyme that liberates one microgram of tyrosine from egg albumin at pH 6.0 and 45° C in 30 minutes.

Effects of certain reducing, oxidising, metallic ions, - SH group blocking and certain other agents on the proteolytic activity of alhagain

5 ml. portions of alhagain solution (5 mg / 100 ml) were mixed with equal volumes of each of the reducing, oxidising, -SH group blocking agents or metallic salt solutions and incubated at 37°C for 3 hours at the end of which the proteolytic activity of the mixture was determined. A control experiment was run under identical conditions by adding 5 ml. of bidistilled water to the enzyme solution.

As the solutions of certain reagents, eg. maleic acid, iodoacetic acid and sodium cyanide were capable of changing the pH of the enzyme solution, the pH of these reagent solutions was adjusted to 6.0 prior to their addition. As PCMB was not fully soluble in water, it was dissolved in 0.1 *N* sodium hydroxide solution and then made up to the required volume.

Specificity of alhagain on B-chain of oxidised insulin

In order to study the specificity of alhagain, the B-chain of oxidised insulin was used as the substrate. The B-chain was prepared from the crystalline insulin by the performic acid oxidation method.[33]

90 mg. of the substrate dissolved in 18ml. portions of veronal buffer (pH 6.0) were mixed with 30 mg. portions of alhagain and 0.1 ml. of merthiolate in stoppered bottles and incubated at 40° C for 6 hours. After the incubation period, the mixture was mixed with an equal volume of 10% trichloroacetic acid, warmed on a boiling water bath and cooled. The resulting precipitate was removed by centrifugation and washed with 5% trichloroacetic acid solution. The washed liquor was added to the main supernatant and the mixture was extracted several times with equal volumes of ahydrous ether to remove trichloroacetic acid. The aqueous solution was made upto a known volume with distilled water. In order to find out the bonds that are specifically cleaved, an aliquot of this solution was used for the determination of free amino acids and N-terminal amino acids of peptides and the remaining solution was used for the C-terminal amino acids of peptides by the procedures as described by Dhar and Bose.[34]

Specificity of alhagain on synthetic substrates

In order to study the specificity of alhagain on certain synthetic substrates, the modified alcohol titration method of Grassmann and Heyde[35] was followed. Each of the synthetic substrates was incubated with alhagain (10:1) for 24 hours at 37° C at pH 6.0. After the period, an aliquot from the digestion mixture was titrated against 0.01 *N* alcoholic potassium hydroxide using 0.1% alcoholic thymolphthalein as the indicator. In each case, a control was run in an identical manner except that boiled enzyme was used in place of alhagain. The extent of hydrolysis was calculated from the net titre value and expressed as relative enzyme activity.

Results

The data obtained with regard to the effect of oxidising, reducing, specific —SH group blocking and other agents on the enzymic activity of alhagain are presented in Table 1.

The various reducing agents that have been studied were found to enhance the proteolytic activity of the enzyme to different degrees. Among the oxidising agents, potassium permanganate and iodine

were observed to inactivate the enzyme, while iodoacetic acid and maleic acid were found to inactivate the enzyme partially. The enzyme was completely inactivated by hydrogen peroxide. DFP, the serine inhibitor, was found to have no effect on the activity of alhagain. Ovomucoid, which is a specific inhibitor of trypsin, was found to inhibit the enzyme activity partially. Urea was found to increase the proteolytic activity of the enzyme. PCMB was found to completely inhibit the activity of alhagain. The metal chelating agents, viz. 8-hydroxy quinoline, *O*-phenanthrolin hydrate and EDTA were found to enhance the proteolytic activity of alhagain. Resorcinol and benzoquinone were observed to inactivate the enzyme completely.

Results obtained with respect to the metallic ions on the activity of alhagain are presented in Table 2.

Out of the various metallic salts that have been studied, manganese chloride, copper sulphate, silver nitrate, magnesium chloride and calcium chloride were found to activate the enzyme. No significant effect was observed in the cases of zinc chloride, ammonium sulphate, disodium hydrogen phosphate and potassium dihydrogen phosphate. A slight degree of inactivation was observed with regard to lead and nickel chlorides. Chlorides of mercury and sodium inhibited the enzyme considerably while ferric chloride inhibited completely.

The different amino acids liberated from the B-chain of oxidised insulin by the action of alhagain are presented in Table 3 and the main sites at which cleavage occured in 6 hours of hydrolysis are indicated by arrows in Fig. 1.

TABLE 1

Effects of certain reducing, oxidising, –SH group blocking and other agents on alhagain

Reagent added (25 mM)	*Activity (% of control)*
Glutathione	1760.8
Ascorbic acid	1474.0
Sodium thiosulphate	328.7
Thioglycollic acid	292.2
Sodium bisulphite	174.8
Sodium cyanide	153.9
Sodium sulphide	151.3
Potassium Permanganate	54.8
Iodine	20.0
Iodoacetic acid	81.3
Maleic acid	75.7
Hydrogen Peroxide	0
DFP	100
Ovomucoid	100
Urea	133
PCMB	0
8-hydroxy quinoline	3992.2
O-phenanthrolin hydrate	381.6
EDTA	167.0
Resorcinol	0
Benzoquinone	0

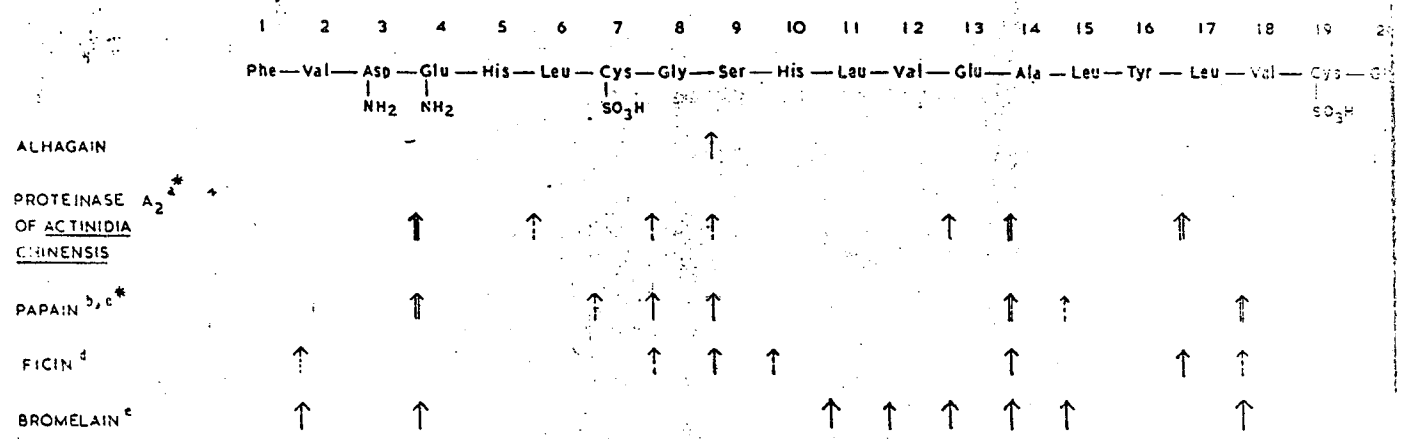
TABLE 2

Effects of certain metallic salts on alhagain

Reagent added (25 mM)	*Activity (% of control)*
Manganese chloride	394.6
Copper sulphate	297.4
Silver nitrate	185.2
Magnesium chloride	153.9
Calcium Chloride	151.3
Zinc chloride	100.0
Disodium hydrogen phosphate	107.8
Potassium dihydrogen phosphate	104.3
Ammonium sulphate	98.7
Lead chloride	83.5
Nickel chloride	79.6
Mercuric Chloride	49.6
Sodium chloride	31.3
Ferric chloride	0

Fig. 1: Comparison of the action of alhagain with those of other plant proteases on the B-chain of oxidised insulin

The sites of cleavage of peptide bonds are indicated by arrows. * The strong, intermediate and weak cleavages are denoted by double, single and broken arrows respectively. a, b, c, d & e represent the results reported by Mc Dowall[15], Johansen *et al.*[45], Johansen and Ottesen[46], Englund *et al*[31] and Kortt *et al*[18] respectively.



a—Mc Dowall, M. A., *Biochim. Biophys. Acta.*, 293, 226 (1973).
b—Johansen, J. T., Ottesen, M., Svendsen, I. & Wybrandt, G., *Compt. Rend. Trav. Lab. Carlsberg*, 36, 365 (1968).
c—Johansen, J. T., Ottesen, M., *Compt. Rend. Trav. Lab. Carlsberg*, 36, 265 (1968).
d—Englund, P. T., King, T. P., Craig, L. C. & Walti, A., *Biochem.*, 7, 163 (1968).
e—Kortt, A. A., Hinds, J. A. & Zerner, B., *Biochem.*, 13, 2029 (1974).

TABLE 3

Yields of free amino acids and N- and C- terminal amino acids of peptides after the hydrolysis of B-Chain of oxidised insulin by alhagain for 6 hours (Results are uncorrected for losses and are expressed as millimoles / 100 g. protein)

Amino acids	*Free*	*N-terminal*	*C-terminal*
Alanine	0 . 83	0	0 . 63
Phenyl alanine	6 . 40	9 . 30	5 . 50
Serine	0	0 . 47	0
Lysine	0	0	0 . 67
Glycine	0	0	2 . 70
Total	7 . 23	9 . 77	9 . 50

The results obtained with regard to the action of alhagain on all the synthetic substrates studied are presented in Table 4.

TABLE 4

Action of alhagain on synthetic substrates

Synthetic substrates	*Enzyme activity**
N-CBZ-L-Glutamyl-L-Tyrosine	++
BAPNA	++
BAA	+
BAEE	+
BAME	–
ATEE	–
CBZ-Glycyl-L-Tryptophan	–
N-Acetyl-L-Tyrosine amide	–

*The relative activity is expressed as ++Good; + moderate and – none.

Discussion

As can be seen from Table 1, all the reducing agents studied were found to enhance the activity of alhagain, out of which glutathione and ascorbic acid produced maximal activation. Glutathione and ascorbic acid were found to be natural activators of papain[25] and ficin[24] respectively. However, Sugiura and Sasaki[6] reported that ascorbic acid had no effect and thiosulphate had inhibitory effect on the activity of ficin. Glutathione was reported to activate the barley acid proteinase[27]. Alhagain resembles asclepain which was found to be activated by cyanide[5], papain and euphorbain which were reported to be activated by cyanide and sulphite[28], proteases from germinated ragi (*Eleusine coracana*)[29] and actinidin[30] both of which were activated by cyanide and sulphide. Cyanide was found to activate both pinguinain[31] and stem bromelain.[17] Alhagain differs from phaseolain which was found to be not activated by reducing agents[14].

Results obtained with respect to the action of oxidising agents on alhagain have indicated (Table 1) that all the oxidising agents inhibited the enzyme. Among the oxidising agents studied, hydrogen peroxide inactivated alhagain completely, while the enzyme was partially inactivated by the action of iodoacetic acid and maleic acid. The activity of the enzyme was found to be inhibited considerably by the action of potassium permanganate or iodine. Greenberg and Winnick[3] have reported the inhibition of some plant proteases viz., asclepain, bromelain and papain by certain oxidising agents. Other plant proteases which were reported to be inhibited by oxidising agents are ficin[6,16], euphorbain[28], actinidin[30], pinguinain[31] and mexicain.[32]

Since alhagain was found to have been appreciably activated by the action of reducing agents and inhibited by oxidising or – SH group blocking agents, it appears that an – SH group is essential for the activity of alhagain. In this respect, alhagain resembles ficin[6,25], stem bromelain[17], the acid proteinase of barley[27] and the thiol proteinase isolated from

buckwheat seeds[44] which were reported to be specifically inactivated by PCMB.

Since alhagain is not inhibited by DFP (Table 1), it does not belong to the class of serine proteases. Similar results were reported in cases of papain[35], stem bromelain[17] and sorghum acid protease.[9] Ovomucoid, a trypsin inhibitor[46], was found to have no inhibitory effect on the enzyme and hence, alhagain does not seem to have serine at the active site.

Urea was observed to increase the proteolytic activity of alhagain (Table 1). The sulphydryl proteinase from *Phaseolus vulgaris var. Perlicka* was reported to get inactivated by urea under different experimental conditions.

Since the three metal chelating agents studied (Table 1) were found not to inhibit the enzyme activity of alhagain, the possibility of the presence of any metallic cofactor in the active centre of the enzyme is ruled out. The total inhibition exerted by ferric chloride (Table 2) and activation by 8-hydroxyquinoline suggests that the latter removes trace of ferric ions. Activation by *O*-phenanthrolin supports this view. Lack of inhibition by metal chelators was reported in the cases of sorghum acid protease,[9] phaseolain[14] and agave protease.[10] Alhagain resembles actinidin[20] and bromelain,[21] since enzyme activity in both the cases was reported to be enhanced in the presence of EDTA.

Resorcinol and benzoquinone were observed to inactivate alhagain completely. These reagents were also reported to inhibit the proteolytic activity of papain.[38]

On studying the effects of various metals on the activity of alhagain, manganese chloride, copper sulphate, silver nitrate, mangnesium chloride and calcium chloride were found to activate the enzyme considerably, while disodium hydrogen phosphate, zinc chloride, ammonium sulphate and potassium dihydrogen phosphate caused no significant effect on the activity of alhagain. Lead chloride, nickel chloride, mercuric chloride and sodium chloride caused significant inhibition in enzymic activity. Alhagain was found to be completely inhibited by ferric chloride (Table 2). Papain and asclepain were reported to get inhibited by Ni^{++}, Co^{++}, Zn^{++}, Fe^{++}, Pb^{++}, Mn^{++} and Fe^{+++}.[39] Alhagain resembles stem bromelain[17] and ficin[8] since both these enzymes were reported to be inhibited by mercuric chloride. The sorghum acid protease was reported to get inhibited by Hg^{++}, Ni^{++} and Fe^{+++}.[9] Alhagain also resembles the sulphydryl proteinase isolated from *Phaseolus vulgaris var. Perlicka* which was found to be inactivated by mercury compounds and activated by calcium compound.[11] The partial inhibition of alhagain by sodium chloride may be compared with zingibain which was also reported to be inhibited by sodium chloride[40].

As can be seen from Table 3, after the hydrolysis of B-chain of oxidised insulin by alhagain for 6 hours, only 2 free amino acids, viz. alanine and phenyl alanine and peptides having 2 N-terminal amino acids, viz. phenyl alanine and serine and 4 C-terminal amino acids, viz. alanine, phenyl alanine, lysine and glycine were liberated. The main sites at which cleavage occured in the B-chain of oxidised insulin by the action of alhagain in 6 hours of hydrolysis are indicated in Fig. 1. Alhagain hydrolyses peptide bonds at residues 8-9, 23-24, 24-25 and 29-30 in 6 hours, releasing peptides and free amino acids. The sites at which cleavage occured in the B-chain of oxidised

insulin by the action of certain other plant proteses are also indicated by arrows in Fig. 1.

Stem bromelain was found to hydrolyse glucagon extensively either at the arginyl - alanyl or alanyl - glutaminyl bond.

On comparing the amounts of free amino acids or N- and C- terminal amino acids of peptides liberated in 6 hours hydrolysis against the total content of the corresponding amino acids[41] occuring in the B-chain of oxidised insulin, it appears that phenyl alanine and glycine are liberated in higher amounts, while smaller quantities of alanine, lysine and serine are also liberated. Alhagain appears to preferentially attack bonds involving the amino or carboxyl groups of phenyl alanine, amino groups of alanine and serine and also carboxyl groups of lysine and glycine.

Results presented in Table 4 indicate that N-CBZ-L - Glutamyl - L-Tyrosine and BAPNA were readily hydrolysed by alhagain. BAA and BAEE were moderately hydrolysed whereas BAME, ATEE, CBZ - Glycyl - L - Tryptophan and N - Acetyl L - Tyrosine amide were not hydrolysed by alhagain.

BAA was found to be the best substrate for the action of papain.[42] BAME was found to be easily hydrolysed by papain,[43] whereas N - Benzoyl - L-tyrosine ethyl ester was not found to be hydrolysed by papain, chymopapain and papaya peptidase A.[44] BAA and BAEE were reported to be easily hydrolysed by ficin.[45] Fruit bromelain was found to hydrolyse BAA and BAPNA greatly. The fruit enzyme was relatively much more active against BAA than stem bromelain.[46] The Protease from *Actinidia Chinensis* was found to catalyze the hydrolysis of BAEE.[47] The trypsin-like proteinase isolated from buckwheat seeds was shown to hydrolyse BAPNA and the ethyl ester of the arginine derivative, but not Benzoyl - tyrosine ethyl ester.[47] ATEE was found to be a good substrate for agave protease but not any derivative of arginine like BAEE, etc.[48] Smith and Kimmel[48] reported that the best substrate for ficin is BAA or BAEE. The best synthetic substrates for papain are derivatives of Benzoyl arginine or lysine.[48] Stem bromelain was reported to hydrolyse BAEE and BAA effectively and was found to have no action on CBZ-L-Glutamyl-L-Tyrosine and ATEE.[47] In this respect, alhagain resembles bromelain for its inability to hydrolyse ATEE. However, it differs from bromelain since it is capable of hydrolysing CBZ-L - Glutamyl-L-Tyrosine.

Acknowledgement

One of the authors (K.Y.) is indebted to the Council of Scientific and Industrial Research, New Delhi, for the award of a research fellowship.

REFERENCES

1. Krishnamurti, C. R. & Subrahmanian, V., *Ind. J. Dairy Sci.*, 2, 19 (1949).

2. Bose, S. M. & Madhavakrishna, W., *Enzymol.*, 19, 186 (1958).

3. Greenberg, D. M. & Winnick, T., *J. Biol. Chem.*, 135, 761 (1940).

4. Balls, A. K. & Lineweaver, H., *J. Biol. Chem.*, 130, 669 (1939).

5. Balls, A. K. & Lineweaver, H., *Nature*, 144, 513 (1939).

6. Skelton, G. S., *Enzymol.*, 35, 279 (1968).

7. Polgar, L., *Eur. J. Biochem.*, 33, 104 (1973).

8. Sugiura, M. & Sasaki, M., *Biochim. Biophys. Acta*, 350, 38 (1974).

9. Garg, G. K. & Virupaksha, T. K., *Eur. J. Biochem.*, 17, 4 (1970).

10. Du Toit, P. J., *Biochim. Biophys. Acta*, 429, 895 (1976).

11. Vavreinova, S. & Turkova, J., *Biochim. Biophys. Acta*, 403, 506 (1975).

12. Mc Dowall, M. A., *Eur. J. Biochem.*, 14, 214 (1970).

13. Dixon, M. & Webb, E. C., *Enzymes*, Longmans, Green and Co. Ltd., London, 272 (1960).

14. Wells, J. R. E., *Biochem. J.*, 97, 228 (1965).

15. Mc Dowall, M. A., *Biochim. Biophys. Acta*, 293, 226 (1973).

16. Jones, L. K. & Glazer, A. N., *J. Biol. Chem.*, 245, 2765 (1970).

17. Murachi, T. & Neurath, H., *J. Biol. Chem.*, 235, 99 (1960).

18. Kortt, A. A., Hinds, J. A. & Zerner, B., *Biochem.*, 13, 2029 (1974).

19. Garg, G. K. & Virupaksha, T. K., *Eur. J. Biochem.*, 17, 13 (1970).

20. Yeshodha, K., Dhar, S. C. & Santappa, M., *Ital. J. Biochem.*, 26 (3), 169 (1977).

21. Yeshodha, K., Dhar, S. C. & Santappa, M., *Ital. J. Biochem.*, 26 (3), 181 (1977).

22. Sanger, F. & Thompson, E. O. P., *Biochem. J.*, 53, 353, 366 (1953).

23. Dhar, S. C. & Bose, S. M., *Enzymol*, 28, 89 (1964).

24. Grassmann, W. & Heyde, W., *Z. Physiol. Chem.*, 183, 32 (1929).

25. Fraenkel, M. & Maimin, R., *Nature*, 140, 1015 (1937).

26. Krishnamurti, C. R. & Subrahmanian, V., *Ind. J. Dairy Sci.*, 2, 43 (1949).

27. Popov, M. P. & Karpilenko, G. P., *Prikl. Biokhim. Microbiol.*, 11 (5), 757 (1975) through *Chem. Abs.* 84, 1598 X (1976).

28. Tauber, H., *The Chemistry and Technology of Enzymes*, John Wiley and Sons, Inc., Second Printing, New York, 160, 167, 173 (1950).

29. Chandrasekhara, M. R. & Swaminathan, M., *J. Sci. Ind. Res.* (India), 12B, 481 (1953).

30. Arcus, A. C., *Bicohim. Biophys. Acta*, 33, 242 (1959).

31. Asenjo, C. F. & Fernandez, C. D., Science, 95, 49 (1942).

32. Castaneda, M., Balcazar, M. R. & Gavarron, F. F., *Science*, 96, 365 (1942).

33. Kortt, A. A., Hamilton, S., Webb, E. C. & Zerner, B., *Biochem.*, 13, 2023 (1974).

34. Iordan, A. G. & Belozerskii, M. A., *Vestn. Mosk. Univ., Biol., Pochvoved*, 30 (5), 115 (1975) through *Chem. Abs.*, 84, 86009 d (1976).

35. Kimmel, J. R. & Smith, E. L., *J. Biol. Chem.*, 207, 515 (1954).

36. Lineweaver, H. & Murray, C. W., *J. Biol. Chem.*, 171, 565 (1947).

37. Li-Pen, C. & Liener, I. E., *Biochem. Biophys. Res. Commun.*, 27, 100 (1967).

38. Bahadur, K. & Atreya, B. D., *Enzymol.*, 21, 238 (1960).

39. Winnick, T., Davis, A. R. & Greenberg, D. M., *J. Gen. Physiol.*, 23, 275 (1940).

40. Thompson, F. H., Wolf, I. D. & Allen, C. E., *J. Food Sci.*, 38 (4), 652 (1974).

41. Sanger, F. & Tuppy, H., *Biochem. J.*, 49, 463-481 (1951).

42. Bergmann, M. & Fruton, J. S., *Science*, 86, 496 (1937).

43. Mc Donald, C. & Balls, A. K., *Federation Proc*, 13, 262 (1954).

44. Schack, P., *Compt. Rend. Trav. Lab. Carlsberg*, 36, 67 (1967).

45. Gutfreund, H., *Faraday Soc. Disc.*, 20, 167 (1955).

46. Ota, S., Moore, S. & Stein, W. H., *Biochem*, 3, 180 (1961).

47. Belozerskii, M. A., Emtseva, I. B. & Kursanova, T. A., *Khim. Proteoliticheskikh Fermentov, Mater. Vses. Simp.* 99 (1973) through *Chem. Abs.* 82, 166385 X (1975).

48. Smith, E. L. & Kimmel, J. R., *The Enzymes*, 4, Ed. by Boyer, P. D., Lardy, H. & Myrback, K., 133 (1960).

49. Johansen, J. T., Ottesen, M., Svendsen, I. & Wybrandt, G., *Compt. Rend. Trav. Lab. Carlsberg*, 36, 365 (1968).

50. Johansen, J. T. & Ottesen, M., *Compt. Rend. Trav. Lab. Carlsberg*, 36, 265 (1968).

51. Englund, P. T., King, T. P., Craig, L. C. & Walti, A., *Biochem*, 7, 163 (1968).