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Isoelectric Point Determination of Immobilized Enzymes Employing Polyanionic and Polycationic Enzyme Inhibitors as Tools

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ABSTRACT

A simple method based on the principle of Wills has been developed for the isoelectric point (pI) determination of immobilized enzymes. This approach has been applied in the case of five alkylamine immobilized enzymes, i.e., sweet potato β-amylase, *B. subtilis* α(-amylase, yeast invertase, bacterial dextranase and *Trichoderma* cellulase employing both polyanionic inhibitors like suramin, inorganic polyphosphates, dextran sulphate and polyanethol sulphonate and polycationic inhibitors like poly-L-arginine, poly-L-ornithine, and poly-L-histidine. The overall competence limitations of the method have been discussed.

INTRODUCTION

During the course of our studies on the in vitro activation, inhibition, and kinetics of α- and β-amylases (both soluble and insoluble), we wanted to characterize these enzymes in both the forms through their isoelectric point (pI) also apart from Km, Vm, Ea, and so on. However, a review of literature (1–4) revealed that no attempt has been made on the pI determi-

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nation of immobilized enzymes, since the physicochemical methods for pI determination were applicable only to soluble enzymes and proteins. Since our studies on enzyme activation and inhibition came under the purview of macroionic interaction, a close survey of literature revealed that a chemical inhibition method that was successfully applied to several soluble enzyme systems for their pI determination employing polyanionic inhibitors like suramin has been developed by Wills (*1*). Since a detailed study has been carried out by us on the activation/inhibition of amylases, invertase, dextranase, and cellulase by polyations (*6–7*), the principle of Wills was applied for the pI determination of the five immobilized enzymes, and the results of these findings are presented in this paper.

MATERIALS AND METHODS

The sources of the biochemicals are mentioned below in parenthesis. Sweet potato β-amylase (E. Merck, W. Germany), yeast invertase (Fluka, Switzerland), *B. Subtilis-α-amylase* (Fluka, Switzerland), dextranase (K. L. Donavan, USA), *Trichoderma* cellulase (Mary Mandels, USA), starch and glutaraldehyde (E. Merck, W. Germany), DNS and PANS (Koch-Light Laboratories, UK), sucrose (BDH, UK), dextran, and dextran sulphate (Sigma, USA), polycations (P. T. Mora, USA), inorganic poly phosphates (U. P. Strauss, USA). All the other chemicals used in this study were of analytical grade.

Immobilization of Enzyme to Zirconia Coated Alkylamine Glass (*8*)

1 g of zirconia coated alkylamine glass was activated by the addition of 10 mL of a 2.5% glutaraldehyde with occasional shaking for 1 h and the excess glutaraldehyde was washed off with water. One hundred mg of each enzyme in phosphate buffer of pH 7.0 (0.05 M) was added to 1 g of activated support and kept overnight for coupling. At the end, the excess enzyme was washed off with an appropriate buffer until no more enzyme activity could be detected in the washings and the immobilized enzymes were stored in their respective buffers at 4°C. They were air dried and used for further experiments.

ASSAY OF IMMOBILIZED ENZYMES

Alkylamine Conjugated Sweet Potato β-Amylase (*9*)

Ten mg of alkylamine conjugated sweet potato β-amylase was employed per reaction mixture and the enzyme was assayed using 2% starch as substrate at 37°C in acetate buffer of pH 4.8 (0.05 M) in a metabolic shaker for 10 min and the reaction stopped by the addition of 1 mL of a 1% DNS reagent and the red color developed was read at 525 mm.

Alkylamine Conjugated *B. subtilis* α-Amylase (*9*)

Fifteen mg of the conjugated α-amylase was employed per reaction mixture and the enzyme was assayed using 2% starch as substrate at 37°C in acetate buffer of pH 5.6 (0.05 M) in a metabolic shaker for 15 min and the maltose liberated was measured by the DNS method.

Alkylamine Conjugated Yeast Invertase (*10*)

Ten mg of alkylamine conjugated invertase was employed per reaction mixture and the enzyme was assayed using 2.5% sucrose as substrate at 37°C in acetate buffer of pH 4.8 (0.05 M) for 5 min in a metabolic shaker with vigorous shaking and the glucose liberated was measured using DNS method.

Alkylamine Conjugated Bacterial Dextranase (*11*)

Twenty mg of the conjugated dextranase was employed per reaction mixture and the enzyme was assayed using 2% dextran as substrate at 37°C in a metabolic shaker in phosphate buffer of pH 6.8 (0.05 M) for 20 min and the glucose released was measured by the DNS method.

Alkylamine Conjugated Trichoderma Cellulase (*12*)

Twenty mg of the conjugated cellulase was employed per reaction mixture and the enzyme was assayed using 2% carboxy methyl cellulose as substrate at 37°C in acetate buffer of pH 4.8 (0.05 M) for 30 min in a metabolic shaker and the glucose released was measured by the DNS method.

Procedure for Determination of pI of an Immobilized Enzyme

The polyanion or polycation in the amount indicated per reaction mixture was preincubated with the respective alkylamine conjugated enzyme for 30 min for causing optimal inhibition. The buffers used were acetate (0.05 M) pH range 3.6–5.6 and phosphate buffer (0.05 M) pH range 5.7–6.8. The effect of pH was studied on the inhibition of each conjugated enzyme caused by a polyion and the midpoint on the pH range where the percent inhibition varied from 0 to 100 was taken as the pI of the enzyme as done by Wills (*1–14*). Protein estimation was carried out according to Lowry (*13*).

RESULTS AND DISCUSSION

Table 1 gives a comparative data on the pI values of both soluble and alkylamine conjugated sweet potato β-amylase obtained employing the six polyanionic inhibitors as tools. An average pI of 4.9 was obtained for the soluble enzyme (*5*) with a deviation of +0.1 employing the six polyanionic

Table 1
Isoelectric Point (pI) Values of Alkylamine Conjugated Sweet Potato β-Amylase Employing Polyanionic Inhibitors as Tools

Polyanion reported pI	Soluble enzyme amt ug/rm	Insoluble enzyme pI	Insoluble enzyme amt ug/rm	Insoluble enzyme pI
	—	4.8	—	—
DS	0.8	4.9	1.0	5.2
PANS	1.0	4.9	2.0	5.3
Suramin	40.0	4.8	50.0	5.1
IPP ($n=90$)	20.0	4.8	50.0	5.1
IPP ($n=110$)	12.0	4.7	15.0	5.3
IPP ($n=190$)	6.0	4.9	8.0	5.2
Av. pI	—	4.8	—	5.2

inhibitors mentioned, the pI reported being 4.8 (*14*). The pI of the alkylamine conjugated β-amylase shifted to 5.2 (by +0.4 U) wherein the pI values obtained employing suramin, PANS, DS, IPPs ($n=90$, 110, and 190) were 5.2, 5.3, 5.2, 5.1, 5.3, and 5.2, respectively. Thus, the overall pI for the alkylamine immobilized β-amylase was shifted from 4.8 for the soluble enzyme to 5.2 for the insoluble enzyme with a shift of +0.4 U (Fig. 1).

Similarly, the six polyanionic inhibitors mentioned above were employed for the pI determination of alkylamine conjugated *B. subtilis* α-amylase and the results are tabulated in Table 2. It is seen from this that the average pI obtained for soluble α-amylase employing these six inhibitors is 5.3 (*5*), whereas the pI reported for the soluble enzyme is 5.4 (*15*). The six polyanionic inhibitors, i.e., DS, PANS, SUR, IPPs ($n=90$, 110, and 190) yielded pI values of 5.9, 5.9, 6.0, 6.1, 6.0, and 6.0 for the alkylamine conjugated α-amylase, the average pI obtained being 6.0. As observed in the case of alkylamine conjugated β-amylase, α-amylase also exhibited a shift in its pI value from 5.4 to 6.0 upon immobilization to alkylamine glass.

In contrast to the two enzymes discussed above, bacterial dextranase did not exhibit any shift in its pI value upon immobilization to alkylamine glass as shown in Table 2. It is seen from this that the pI reported for the soluble enzyme employing suramin as a tool is 4.3 (*5*) and no further shift in its pI value is observed on conjugation to alkylamine glass employing suramin.

Yeast invertase and *Trichoderma* cellulase, in contrast to the three enzymes discussed above got inhibited by polycations, i.e., poly-L-arginine, poly-L-histidine, and poly-L-ornithine and hence, the three polycations were employed for the pI determination of alkylamine conjugated invertase and cellulase.

Table 3 gives a comparative data on the pI values of soluble and alkylamine immobilized yeast invertase obtained employing suramin and poly-

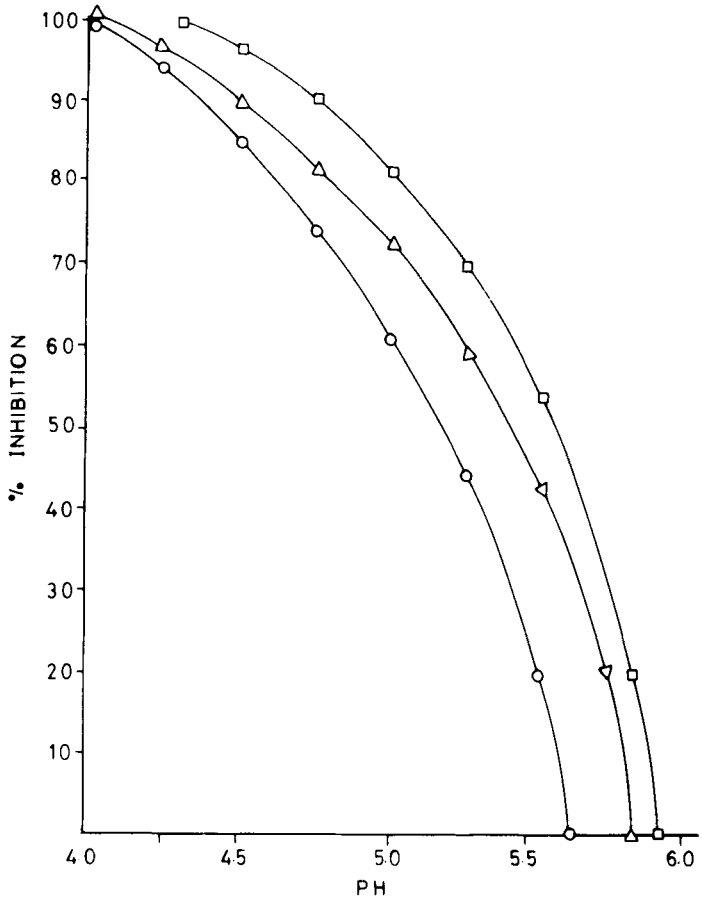

Fig. 1. Effect of pH on the inhibition of soluble sweet potato α-amylase by suramin (●——●), dextran sulfate (△——△), alkylamine immobilized β-amylase by suramin (□——□).

Table 2
Isoelectric Point (pI) Values of Alkylamine Conjugated Bacterial Dextranase and *B. Subtilis* α-(Amylase Employing Polyanionic Inhibitors as Tools

Polyanion	Soluble enzyme	Insoluble enzyme
B. Subtilis α-amylase:		
DS	5.2	5.9
PANS	5.4	5.9
Suramin	5.3	6.0
IPP ($n=90$)	5.3	6.1
IPP ($n=110$)	5.2	6.0
IPP ($n=190$)	5.4	6.0
Average pI	5.3	6.0
Reported pI	5.4	—
Bacterial dextranase		
Reported pI	4.2	—
Sur	4.3	4.3

Table 3
Isoelectric Point Values of Alkylamine Immobilized Yeast Invertase Employing Polyanionic Inhibitors as Tools

Polyion reported pI	Soluble enzyme		Insoluble enzyme	
	amt, ug/rm	pI	amt, ug/rm	pI
Reported pI	—	5.0	—	—
Suramin	65	4.9	100	5.5
Poly-L-histidine	50	5.2	80	5.6
Average pI	—	5.1	—	5.5

L-histidine as tools. A pI of 5.0 has been reported for the soluble enzyme (*17*), whereas 5.1 was obtained as the pI for this enzyme, employing poly-L-histidine as a tool and suramin yielded a value of 4.9. A shift of +0.4 U was obtained in the pI of invertase upon conjugation to alkylamine glass as the average pI obtained was 5.5 for the insoluble enzyme. In contrast to this, no shift in the pI was observed for *Trichoderma* cellulase upon immobilization to alkylamine glass (Table 4, Fig. 2). As may be seen from this table, a pI of 5.3 was obtained by this method for the soluble cellulase, the pI reported being 5.1 (*18*) and no further shift in the pI was observed for this enzyme upon conjugation, as indicated in Table 4. This behavior was similar to that observed for alkylamine conjugated dextranase, where no shift in pI was observed on conjugation either. The shift in the pI values of enzymes upon conjugation to alkylamine glass to higher pHs may be explained by the fact that the pH of the surface of the carrier was more acidic than would be expected owing to the presence of the positively charged or neutrally charged zirconia residues. Unsubstituted amino groups on the zirconia coated alkylamine glass in the immediate environment of the fixed enzyme presumably maintain the pI of alkylamine immobilized dextranase and cellulase.

MECHANISM OF ISOELECTRIC POINT (pI) CHANGE

The immobilization of an enzyme occurs in two steps: Step 1 involves the attachment of glutaraldehyde molecules to the free amino groups of the enzyme and this obviously results in a loss of amino groups (basic groups) of the enzyme and therefore with basicity of the enzyme molecule. In Step 2 the glutaraldehyde alkylamine conjugate (activated support) gets chemically linked to the support enzyme, and, in this process, the alkylamine glass contributes its free amino groups to different extents to different immobilized enzymes.

It is probably the critical/vital free amino groups of the alkylamine glass, which are in the vicinity of the bound enzyme molecule or its operational sphere and which are being contributed toward the enzyme basicity.

Table 4
Isoelectric Point (pI) Values of Alkylamine Conjugated *Trichoderma* Cellulase Employing Polyanionic Inhibitors as Tools

Polyion reported pI	Soluble enzyme		Insoluble enzyme	
	amt, ug/rm	pI	amt, ug/rm	pI
Reported pI	—	5.2	—	—
Poly-L-arginine	60	5.3	80	5.4
Poly-L-ornithine	45	5.2	60	5.3
Poly-L-histidine	30	5.3	50	5.3
Average pI	—	5.3	—	5.3

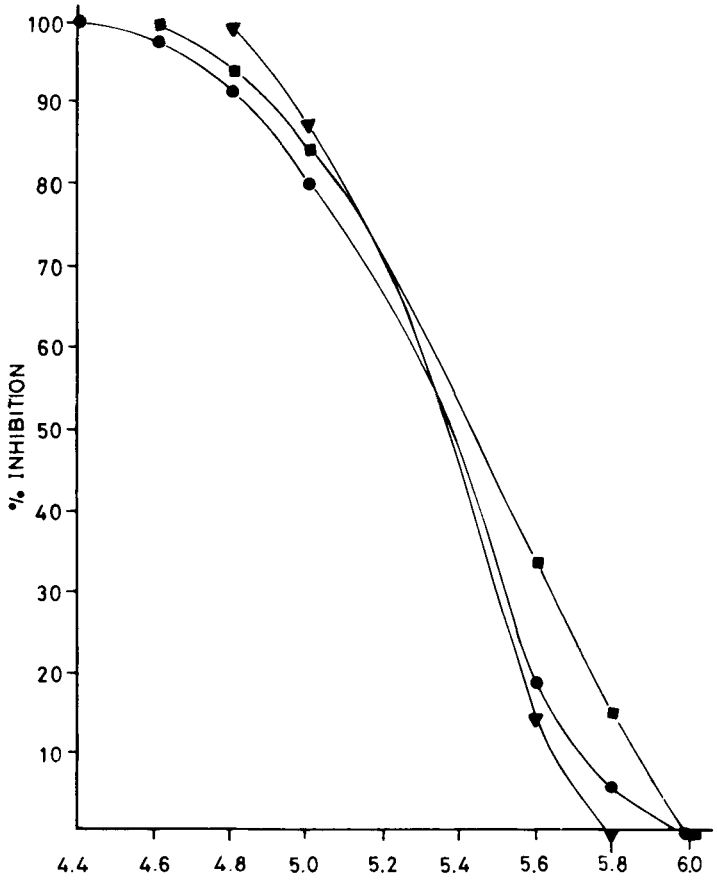

Fig. 2. Effect of pH on the inhibition of *Trichoderma* cellulase (soluble) by poly-L-arginine (▼——▼), poly-L-ornithine (●——●); alkylamine conjugated Cellulase by poly-L-histidine (□——□).

In the case of the two immobilized enzymes (dextranase and cellulase), no change occurred in the pI (compared to the soluble enzyme), and this may be attributed to the loss in basicity (amino groups) being compensated for by the gain in the free amino groups from the alkylamine glass

matrix. However, with the second group comprising α-amylase, β-amylase, and invertase, which display a shift (i.e., increase) in pI values (to different extents in each case), it may be attributed to the loss in amino groups in Step 1 being less than the amino groups in Step 2.

CONCLUSIONS

The principle of Wills has been greatly enlarged in its scope by

1. Extending it to five immobilized enzyme systems;
2. Reporting eight new polyionic inhibitors as tools for the pI determination of enzymes;
3. Applying the principle of Wills in the converse by employing three polycationic inhibitors as tools; and
4. Affording a new simple method for characterizing immobilized enzymes through their pI values where conventional techniques like isoelectric focusing fail owing to their insoluble nature.

This method is being recommended for enzymes (soluble and insoluble) undergoing inhibition by polyions (*19–22*).

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REFERENCES

1. Wills, E. D. and Wormall, A. (1950), *Biochem. J.* **47,** 158.
2. Town, E. W., Wills, E. D., and Wormall, A. (1949), *Nature,* **163,** 735.
3. Wills, E. D. (1952), *Biochem. J.* **50,** 421.
4. Wills, E. D. and Wormall, A. (1950), *Nature* **165,** 813.
5. Ramesh, V. and Chanan Singh, (1979), *Ind. J. Biochem. Biophys.* Suppl., 16, 78.
6. Ramesh, V. and Chanan Singh (1981), *J. Mol. Catal.* **10,** 341.
7. Ramesh, V. and Chanan Singh (1980), *Biochem. Biopys. Res. Commun.* **97,** 779.
8. Weetall, H. H. (1975), Immobilized Enzymes, Antigens, Antibodies and Peptides, M. Dekker, New York, p. 245.
9. Bernfeld, P. (1955), in *Methods in Enzymology,* Colowick, S. P. and Kaplan, O., eds, vol. 1, pp. 144–158, Academic, New York.

10. Fisher and Kohtes (1955), in *Methods in Enzymology,* Colowick, S. P. and Kaplan, O, eds. vol. 1, pp. 251–252, Academic, New York.
11. Janson, J. L. and Porath, J. (1966), in *Methods in Enzymology,* Colowick, S. P. and Kaplan, O. eds. vol. 8, pp. 615–617, Academic, New York.
12. Stevens, G. (1955), in *Methods in Enzymology,* Colowick, S. P. and Kaplan, O., eds., vol. 1 pp. 149–158, Academic, New York.
13. Lowry, O. H., Rosenbrough, N. H., Farr, A. L. and Randall, R. J. (1951), *J. Biol. Chem.* **193,** 265.
14. Englard, S. and Singer, T. P. (1950), *J. Biol. Chem.* **187,** 213.
15. Junge, J. M. and Fischer, E. H. (1959), *J. Biol. Chem.* **234,** 556.
16. Personal Communication from K. L. Donavan, Beckman Labs, USA (1978).
17. Hestrin, S., Feingold, D. S. and Schram, N. (1955), in *Methods in Enzymology,* Colowick, S. P. and Kaplan, O., eds., vol. 1, pp. 256–257, Academic, New York.
18. Personal Communication from Mary Mandels, Natick, USA (1978).
19. Elbein, A. D. (1974), in *Advances in Enzymology,* Meister, A., ed., pp. 29–64, Wiley, London.
20. Ramesh, V. and Chanan Singh. (1982), *J. Appl. Biochem.,* **4,** 81.
21. Ramesh, V. and Chanan Singh. (1981), *Enz. Microb. Technol.* **3,** 246.
22. Righetti, P. G. and Caravaggio, T. (1976), *J. Chromatog.* **127,** 1.