Biochimica et Biophysica Acta, 445 (1976) 169—181

BBA 67887

SPECIFIC IRREVERSIBLE INHIBITION OF SWEET-ALMOND β-GLUCOSIDASE BY SOME β-GLYCOPYRANOSYLEPOXYALKANES AND β-D-GLUCOPYRANOSYL ISOTHIOCYANATE

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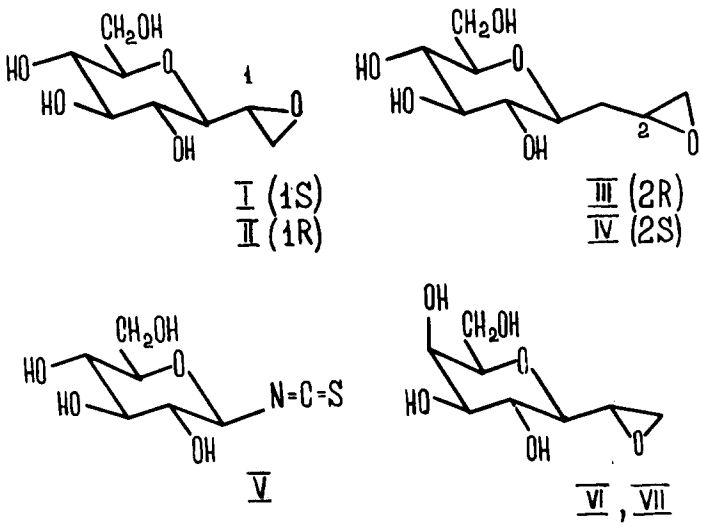
(Received January 27th, 1976)

Summary



β-D-Glucopyranosyl-(1S and 1R)-epoxyethanes (I and II), 1-(β-D-glucopyranosyl)-(2R and 2S)-2,3-epoxypropanes (III and IV), β-D-glucopyranosyl isothiocyanate (V) and β-D-galactopyranosylepoxyethane (VI) are active-site-directed irreversible inhibitors of sweet-almond β-glucosidase B (β-D-Glucoside glucohydrolase, EC 3.2.1.21). Formation of the covalent bond is preceded by the binding of these inhibitors in the active site of the enzyme. This is testitified by the competitive character of inhibition of β-glucosidase component B by compounds I—VI at the early period and by the protection of the enzyme from inactivation by its competitive inhibitors D-glucose and 1,5-D-gluconolactone.

Epoxides I—IV are bound covalently with component B at a molar ratio

1 : 1 as shown with the aid of ^{14}C-labelled inhibitors. The release of the label from modified enzyme ($E \cdot I_{covalent}$) by treatment with hydroxylamine suggests the formation of an ester bond between inhibitors I—IV and the carboxyl group of the enzyme active site. The pH dependence curve of the inactivation rate of β-glucosidase B is of a bell-shaped form for V and of a sigmoid character for I—IV and points to the involvement of the active site groups with pK_a 5.6—5.9 and 4.2—4.4.

Introduction

The method of affinity labelling enabled us to identify peptides directly incorporated into the active sites of a number of enzymes [1,2]. In the case of esterases and proteases the synthesis of the required irreversible inhibitors is a rather routine procedure as the specific inactivation of these enzymes may be frequently achieved either by the use of structurally affinitive or the very same inhibitors (for example, diisopropylfluorophosphate and related compounds).

However, *O*-glycoside hydrolases (EC 3.2.1.-), enzymes hydrolyzing *O*-glycosidic bonds, owing to the high substrate specificity, need, for each glycosidase, an inhibitor of a particular structure. As a rule the synthesis of such compounds is a difficult problem. Possibly for this reason the study of *O*-glycoside hydrolases with the aid of irreversible inhibitors has been applied only recently.

There has been achieved an irreversible inhibition of active sites of such endo-glycanases as lysozyme [3,4] and cellulases [5], and such glycosidases as β-glucosidases [6—8], β-galactosidase [9,10], saccharase and isomaltase [11]. So far there are no descriptions of irreversible inhibition of exo-glycanases.

One may assume that the synthesis route of irreversible inhibitors of endo-glycanases developed by Thomas [12], Sharon et al. [3,4], and Legler and Bause [5] is of a common pattern. This route is based on the attachment of the alkyl chain carrying the nucleophylic group at the respective oligosaccharides (Fig. 1A).

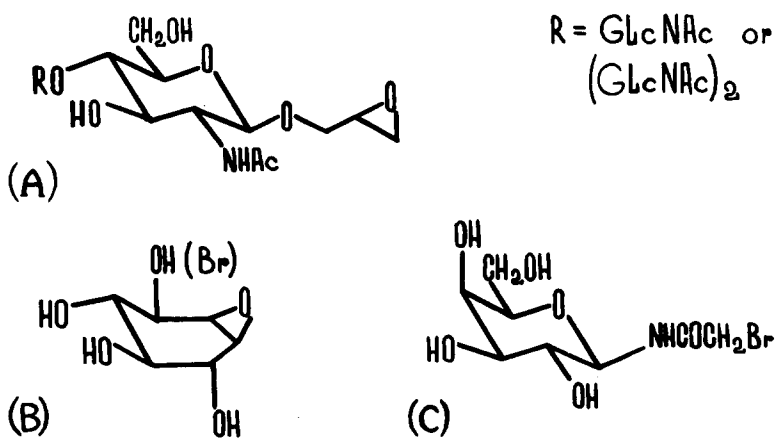


Fig. 1. Irreversible inhibitors of lysozyme (A) [3,12], β-glucosidase (B) [6,7] and β-galactosidase (C) [9].

In the case of glycosidases the choice of irreversible inhibitors is rather limited. Indeed, the highly specific inhibitors described by Legler [6—8] (Fig. 1B) are suitable only for inactivation of enzymes splitting off the glucose residues (cf. ref. 11). Further the irreversible inhibition of β-galactosidase by *N*-bromoacetyl-β-D-galactopyranosylamine (Fig. 1C) [9] has not led to the labelling of a catalytic group of this enzyme [10].

However, the possibility of obtaining glycosidase inhibitors from available monosaccharides appears to be encouraging.

Bearing in mind the high specificity of glycosidases to the glyconic (splitting off) part of the substrate one may formulate the following basic requirements to active-site-directed irreversible inhibitors of glycosidases capable of interaction with the enzyme catalytic groupings: (a) They must provide the necessary orientation of an inhibitor in the enzyme active site. Thus glyconic parts of a substrate and an inhibitor should reveal maximum similarity. (b) The inhibitor grouping able to form a covalent bond in an active site must be directly connected with C_1 atom of the monosaccharide residue so that this group lies close to the enzyme catalytic grouping within the enzyme-inhibitor complex. (c) The irreversible inhibitor should not be subject to enzymatic hydrolysis. (d) The newly formed covalent bond ought to be sufficiently stable.

Inhibitors meeting these demands may, in particular, be found among C- and N-glycosides.

Earlier we described the synthesis and some properties of diastereomeric β-D-glucopyranosylepoxyethanes (I, II), 1-(β-D-glucopyranosyl)-2,3-epoxypropanes (III, IV) [13], β-D-glucopyranosyl isothiocyanate (V) [14], and diastereomeric β-D-galactopyranosylepoxyethanes (VI, VII) [15] (Fig. 2).

This paper is devoted to the study of the interaction of compounds I—VII with component B of sweet-almond β-glucosidase (β-D-Glucoside glucohydrolase, EC 3.2.1.21).

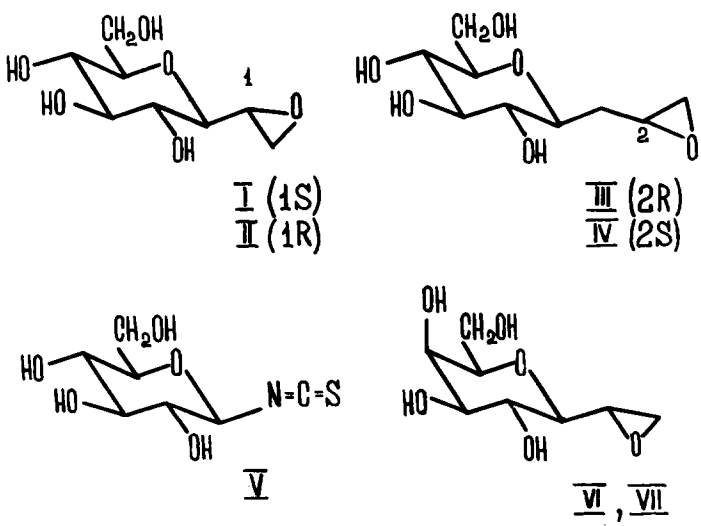
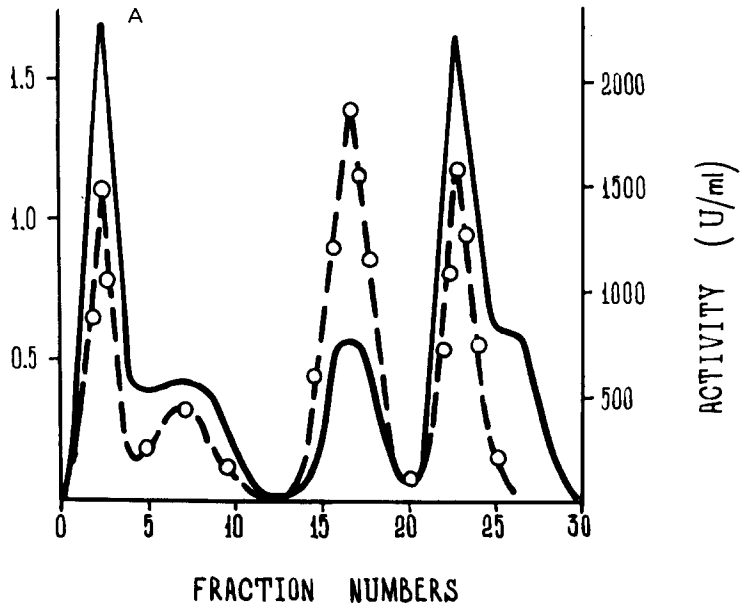


Fig. 2. Irreversible inhibitors of sweet-almond β-glucosidase [13—15].



Materials and Methods

Enzyme. 100 mg of commercial sweet-almond β-glucosidase (specific activity 1000 units/mg of protein, Calbiochem, Lot 000689, U.S.A.) was purified by column chromatography on DEAE cellulose (0.7 × 13 cm, Whatman, England) (Fig. 3A). Fractions NN 21—24, containing component B [16], were dialyzed against distilled water, lyophilized and chromatographed on Sephadex G-200 (0.8 × 40 cm, Pharmacia, Sweden) (Fig. 3B). The fractions obtained, NN 3—6 were desalinized by dialysis and lyophilized; as a result 10 mg of compinent B, ($E^{1\%}_{280}$ = 17.5, cf. ref. 17), with a specific activity 3000 units/mg of protein, was obtained (Table I).

Substrates and inhibitors. *p*-Nitrophenyl-β-D-glucopyranoside, m.p. = 164—165°C, $[\alpha]^{20}$ = —102° (c1, water), and *p*-nitrophenyl-β-D-galactopyranoside, m.p. = 179—180°C, $[\alpha]^{20}$ = —83° (c1, water), (Chemapol, C.S.S.R.) were recrystallized from alcohol; 1,5-lactone of gluconic acid, m.p. = 148—151°C, $[\alpha]^{20}$ = +60° (c1, alcohol) was obtained according to the method of ref. 18. β-D-Glucopyranosyl-(1S)-epoxyethane * (I), $[\alpha]^{20}$ = —14° (here and further c1, methanol), β-D-glucopyranosyl-(1R)-epoxyethane (II), $[\alpha]^{20}$ = —6°, 1-(β-D-glucopyranosyl)-(2R)-2,3-epoxypropane (III), $[\alpha]^{20}$ = —14°, 1-(β-D-glucopyranosyl-(2S)-2,3-epoxypropane (IV), $[\alpha]^{20}$ = —2°, were obtained according to the method of ref. 13; ^{14}C-labelled inhibitors I—IV were obtained analogously from ^{14}C-labelled D-glucose (Medpreparat Works, U.S.S.R.). β-D-Glucopyranosyl isothiocyanate V **, $[\alpha]^{20}$ = +72°, [14] and diastereomeric β-D-galactopy-

* The absolute configuration of the asymmetric carbon atom of the epoxide ring in compounds I—IV is determined by X-ray crystallographic analysis [19].

** Recently Taverna and Langdon [20] used this compound as an irreversible inhibitor of glucose translocation in the erythrocyte membrane.

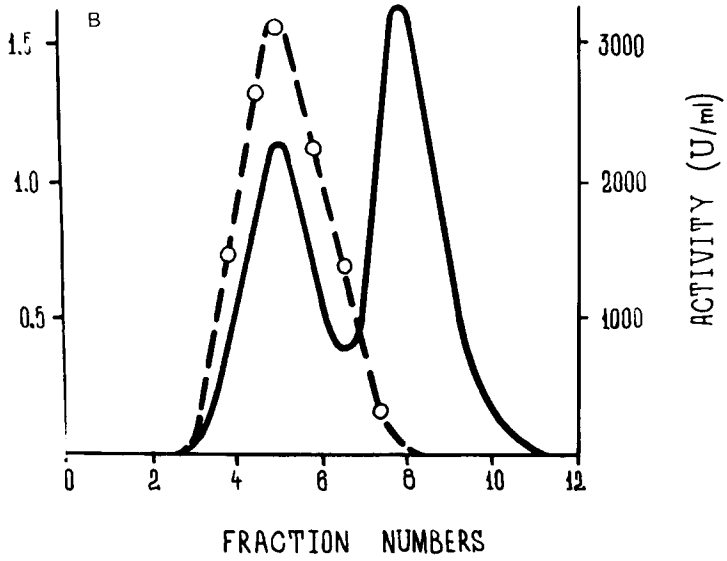


Fig. 3. A. Chromatography of commercial sweet-almond β-glucosidase on DEAE-cellulose (0.7 × 13 cm) equilibrated with 0.005 M acetate buffer (pH 6.2). The protein is eluted with buffer gradients: 0.005 → 0.1 M (100 ml each) and 0.1 → 0.2 M (50 ml each); flow rate 0.7 ml/min, each fraction contains 10 ml; ———, absorption at 280 nm; - - - - - -, glucosidase activity (μmol of *p*-nitrophenol/min/ml). B. Gel filtration of DEAE fractions 21—24 on Sephadex G-200 (0.8 × 40 cm) equilibrated with 0.01 M acetate buffer (pH 6.2). Protein is eluted with the same buffer; flow rate 6 ml/h; each fraction contains 10 ml; ———, absorbance at 280 nm; - - - - - -, glucosidase activity (μmol of *p*-nitrophenol/min/ml).

ranosylepoxyethanes VI, $[\alpha]^{20} = -4°$, and VII, $[\alpha]^{20} = +12°$, [15] were synthesized as described earlier.

Inhibitor stability. Isothiocyanate V is quite stable in citrate/phosphate buffer at 37°C between pH 4.2 and 6.5. Under these conditions epoxides I—IV, VI and VII are unstable; work with them requires buffers not containing carbonic acids.

Buffers. Buffers of ionic strength 0.1 were used: citrate/phosphate (pH 4.0—7.0) in experiments with inhibitor V, barbiturate/phosphate (pH 4.0—6.0) and phosphate (pH 5.8—7.2) in experiments with epoxides I—IV, VI and VII.

Methods. The protein content was estimated spectrophotometrically at 280 nm and according to the method of Louri [21]. All operations involved with the enzyme isolation and purification were carried out at +4°C; when incuba-

TABLE I

ISOLATION OF COMPONENT B FROM COMMERCIAL SWEET-ALMOND β-GLUCOSIDASE

Fractions	Protein content (mg)	Total activity (units)	Specific activity (units/mg)
Initial enzyme	100	100 000	1000
DEAE (21—24)	26	34 000	1300
G-200 (3—6)	10	30 000	3000

tion exceeded 6 h 1 or 2 drops of toluene was introduced into incubation mixture.

Enzymatic activity was determined in citrate/phosphate buffer (pH 6.0) at 37°C; the substrate and enzyme concentrations were 0.02 M and 10—20 μg/ml, respectively. After 10 min incubation the reaction was ceased by adding twice the volume of 0.2 M sodium carbonate solution; the liberated *p*-nitrophenol was determined spectrophotometrically at 400 nm. Specific activity is expressed as μmol of *p*-nitrophenol released per mg protein per min.

The inactivation of β-glucosidase B by applying irreversible inhibitors was carried out as follows: 1 ml of incubation mixture contained $5 \cdot 10^{-2}$ mol of inhibitor and 90 μg (10^{-9} mol) of enzyme in citrate/phosphate (in case V) or barbiturate/phosphate (in the case of I—IV, VI and VII) buffer (pH 5.0) at 37°C. Throughout the indicated time intervals 0.1 ml aliquots were diluted 30 times by the substrate solution, ($[S]_{final}$ = 0.02 M) and the enzymatic activity was estimated.

Radioactivity was measured using a liquid scintillation counter SL-30, Intertechnique (France).

Results and Discussion

Irreversible inactivation of component B. The tests have shown that compounds I—VI irreversibly inactivate sweet-almond β-glucosidase. The changes of component B activity with time is represented in Fig. 4 on semilogarithmic coordinates.

To achieve complere enzyme inactivation the mixture was kept at pH 5.0 from 6 to 48 h (depending on the inhibitor) and the enzyme activity was checked regularly. Under prolonged incubation, owing to the high lability of inhibitors I—VI, the latter were added in portions every 10 h. In control experiments the enzyme activity decreased not more than 10% at 37°C and not more than 5% at 25° for 24 h.

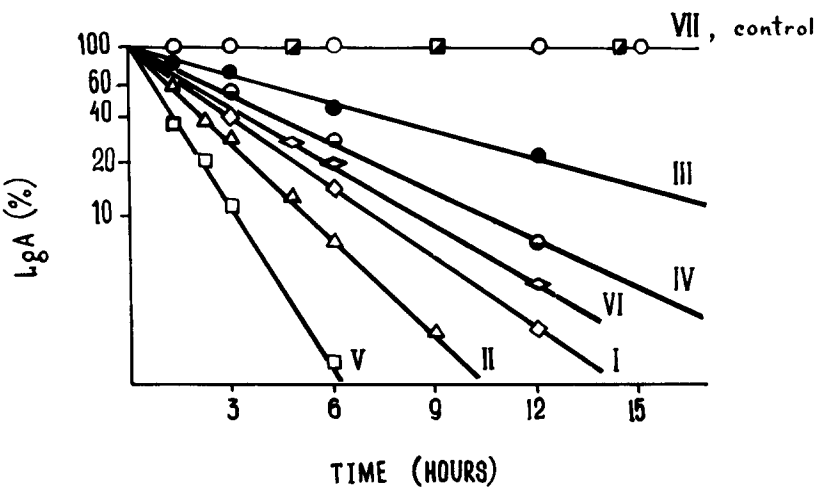


Fig. 4. Decrease with time of the relative activity of component B by applying irreversible inhibitors I—VII at [I] = 50 mM, [E] = 90 μg/ml (pH 5.0, 37°C). ○, control.

Activity of the modified enzyme was not restored either under conditions of exhaustive dialysis or upon gel chromatography on Sephadex G-100.

Under conditions when $[I]_0 \gg [E]$ a linear relationship of inactivation rate of component B upon the inhibitor concentration was observed (Fig. 5). This enabled us to calculate the apparent second order constants k_{+2} ($M^{-1} \cdot min^{-1}$) for enzyme inactivation represented in Table II.

Determination of the character of inhibition of component B. The study of the early stage of interaction of compounds I—VII with component B has shown that in enzymatic hydrolysis of *p*-nitrophenyl-β-D-glucopyranoside these inhibitors behave as competitive ones (Fig. 6). The values of inhibition constants (K_i) determined during the first 10—15 min are displayed in Table II.

Protection of component B with competitive inhibitors from inactivation with compounds I—VI. The rate of the enzyme inactivation by compounds I—VI decreased in the presence of such competitive inhibitors of β-glucosidase as 1,5-D-gluconolactone [22] and D-glucose [23] (Fig. 7). The complete protection of the enzyme from inactivation is achieved at lactone concentration 10^{-3} M, i.e 5 times greater than its K_i, 0.22 mM [24].

Inactivation of β-glucosidase B by ^{14}C-labelled inhibitors I—IV. The stoichiometry of binding of these inhibitors with component B of sweet-almond β-glucosidase was determined with the aid of ^{14}C-labelled compounds I—IV.

2 mg of the enzyme was soluted in 10 ml of barbiturate/phosphate buffer (pH 5.0) and incubated at 25°C with 100 mg of ^{14}C-labelled epoxide (I or II) for 24 h. An additional 100 mg of inhibitor was introduced 10 h after the beginning of incubation. The latter, when carried out with epoxides III and IV, lasted 30—48 h, the inhibitor was added four times in portions to a final concentration of 20 mg/ml. Having attained the desirable degree of inactivation the excess reagent was removed by dialysis with subsequent gel chromatography on

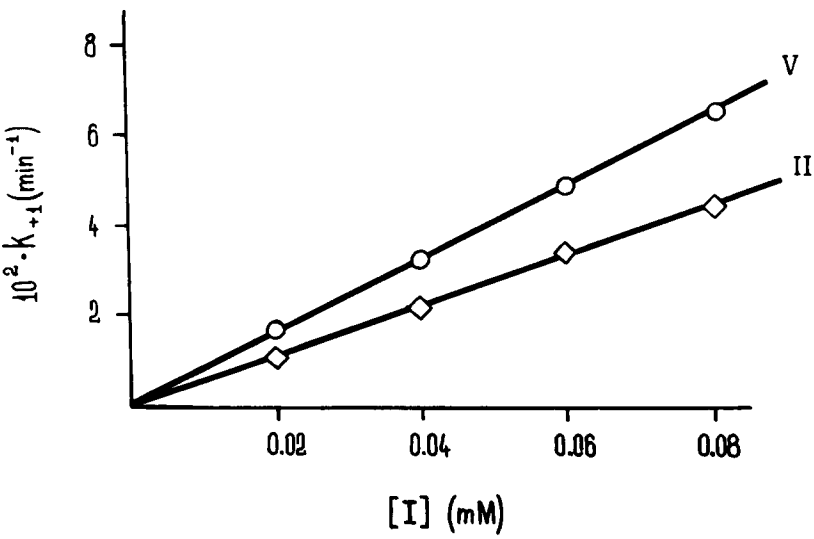


Fig. 5. The dependence of the inactivation rate of component B upon the concentration of inhibitors II and V at pH 6.0 and 37°C.

TABLE II

INHIBITOR CONSTANTS (K_i) AND APPARENT SECOND-ORDER CONSTANTS FOR ENZYME INACTIVATION (k_{+2}) OF COMPOUNDS I—VII

K_i values are calculated from the equation $K_i = K_m/\{tg\alpha(K_m + [S])\}$, where K_m = 1.9 mM (calculated according to the method of Lineweaver and Burk), and α is the slope of the experimental curve (Fig. 6). k_{+2} values are calculated from the equation $k_{+2} = -\{(\ln A/A_0 \cdot [I])/\tau\}$, where A/A_0 is the relative enzyme activity at time τ.

Inhibitor	K_i (M), pH 6.0	k_{+2} ($M^{-1} \cdot min^{-1}$)	
		pH 6.0	pH 5.0
I	0.024	0.05	0.10
II	0.009	0.06	0.15
III	0.048	0.03	0.065
IV	0.035	0.04	0.08
V	0.013	0.08	0.25
VI	—	0.04	0.075
VII	0.04	—	—

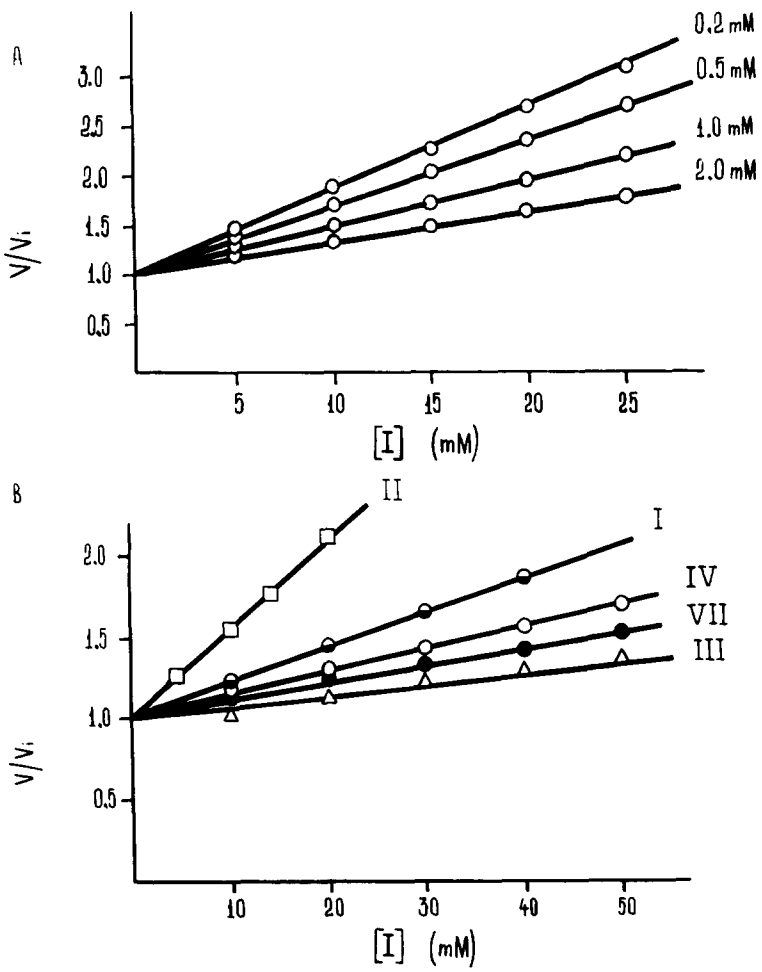


Fig. 6. The V/V_i dependence (pH 6.0, incubation at 37°C for 10 min, [E] = 20 μg/ml) upon concentration (A) of β-D-glucopyranosyl isothiocyanate (V) in a citrate/phosphate buffer at [S] = 0.2—2.0 mM and [I] = 5—20 mM; (B) of epoxides I—IV and VII in a phosphate buffer at [S] = 2 mM and [I] = 5—50 mM. Each point is a result of 3—4 measurements; the calculations were based on the least squares method.

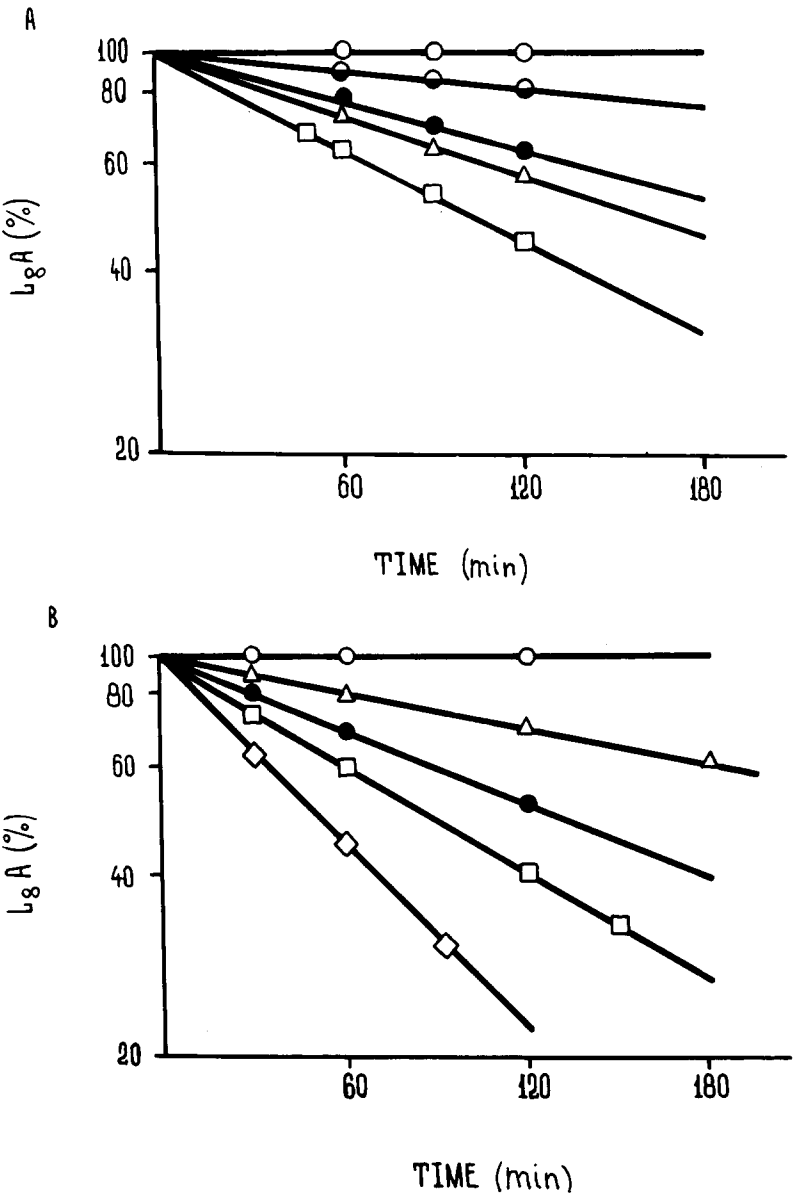


Fig. 7. Protection of component B with competitive inhibitors from inactivation by compounds I—VI at pH 5.0 and 37°C. 1 ml of incubation mixture contained 90 μg (10^{-9} mol) of enzyme. (A) -○-, IV or VI (0.05 M) + lactone (1 mM); -⊖-, III (0.05 M) + lactone (0.1 mM); -●-, III (0.05 M), -△-, III (0.1 M) + glucose (0.1 M); -□-, III (0.1 M). (B) -○-, II or V (0.05 M) + lactone (1 mM); -△-, V (0.05 M) +lactone (0.1 mM). -●-, V (0.025 M) + glucose (0.1 mM); -□-, V (0.025 M); -◇-, V (0.05 M).

Sephadex G-100 (Fig. 8). Fractions NN 3—7 containing ^{14}C-labelled proteins were dialyzed against water (4 × 0.5 l) and lyophilized. As shown in Table III, the incorporation of ^{14}C label nearly coincides with the extent of the enzyme inactivation. A covalent binding of inhibitors with the enzyme develops at 1 : 1 molar ratio.

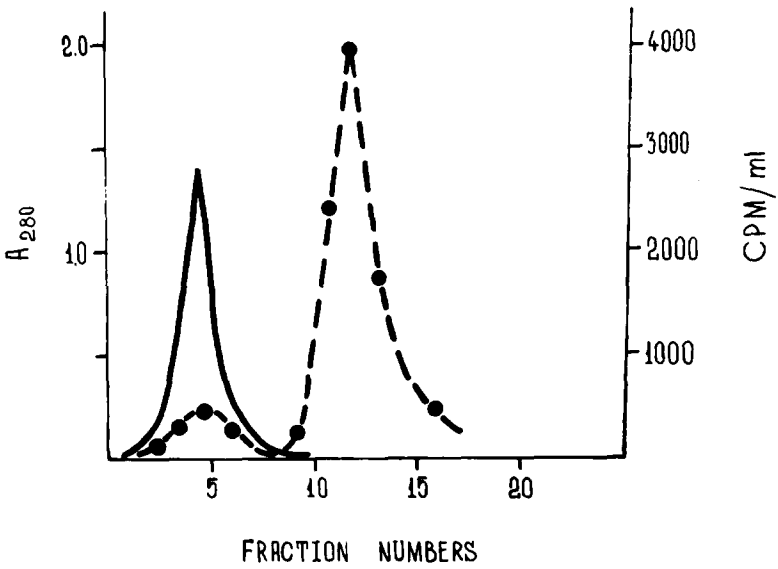


Fig. 8. Gel-filtration of ^{14}C-labelled protein on Sephadex G-100 (0.8 × 50 cm) equilibrated with 0.1 M acetate buffer (pH 6.0). The protein is eluted with the same buffer, flow rate 0.3 ml/min; each fraction contains 5 ml; ———, absorption at 280 nm; - - - - -, radioactivity (cpm).

The label removed by hydroxylamine. The covalent link formed by epoxides I—IV with the group of the enzyme active site turned out to be labile towards hydroxylamine. The solution of 0.8—1 mg ^{14}C-labelled protein in 1 ml of aqueous solution of 0.5 M hydroxylamine hydrochloride was brought up to pH 9.5 by means of Amberlite IRA-400 (OH^-) and incubated for 16 h at 30°C. This was followed by dialysis and lyophilization; the dialyzate was evaporated.

As seen from Table IV, the label had completely transferred into the dialyzate; this fact supports the view that the newly formed covalent bond is of an ester nature (cf. ref. 7).

The results outlined show that compounds I—VI irreversibly inactivate sweet-almond β-glucosidase by covalently binding in its active site. The competitive character of the enzyme inhibition by compounds I—VII and its protection from inactivation in the presence of competitive inhibitors (D-glucose and

TABLE III

INCORPORATION OF LABEL INTO β-GLUCOSIDASE B IN THE COURSE OF ITS INACTIVATION WITH LABELED ^{14}C-INHIBITORS I—IV

Inhibitor	Inactivation (%)	Radioactivity (cpm/mg of protein)	Inhibitor: enzyme (molar ratio)	Inhibitor: $E \cdot I_{covalent}$ (molar ratio)
I	100	1047	0.94	0.94
I	60	622	0.56	0.93
II	100	1089	0.98	0.98
III	79	781	0.70	0.89
IV	60	609	0.55	0.91

TABLE IV

SPLITTING-OFF OF THE LABEL UNDER THE EFFECT OF 0.5 M HYDROXYLAMINE FROM PROTEIN ^{14}C-LABELLED WITH EPOXIDES I—III (pH 9.5, 35°C)

Inhibitor	Fractions	Protein content (mg)	Radioactivity (cpm)	Split-off IV label (%)
I	Protein	0.9	±20	106
	Diazylate	—	997 ± 10	
II	Protein	1.0	±15	96
	Dialyzate	—	1043 ± 26	
III	Protein	0.89	±28	90
	Dialyzate	—	627 ± 19	
IV	Protein	1.6	±21	95
	Dialyzate	—	930 ± 17	

1,5-D-gluconolactone) depending upon their concentration and affinity to the active site confirms the view that the covalent binding of irreversible inhibitors in the active site is preceded by the formation of the enzyme-inhibitor complexes. Thus, the enzyme inactivation may be represented as:

$$E + I \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} E \cdots I \xrightarrow{k_{+2}} E \cdot I_{covalent}$$

When determined with the aid of ^{14}C-labelled I—IV, molar ration E : I in $E \cdot I_{covalent}$ is 1 : 1. This is compatible with the sole active site in the enzyme molecule (mol. wt. = 90 000 [25]). The same result was obtained by Legler while inactivating component B of the sweet-almond glucosidase with conduritol B epoxide [7].

The dependence of component B inactivation rate upon pH. To clarify the nature of the active site groups participating in the formation of covalent bonds we determined the pH dependence of the rate of the enzyme irreversible inhibition by compounds I—V.

The pH dependence curve of the inactivation rate of the enzyme by isothiocyanate V is bell-shaped, similar to the pH dependence curve of hydrolysis rate of *p*-nitrophenyl-β-D-glucopyranoside (Fig. 9). The pK_a values of the groups participating in the process of enzyme inactivation were determined from this dependence after the method of ref. 26 and were approx. 5.6 and 4.4, respectively. At the same time the pH dependence of the enzymatic hydrolysis of *p*-nitrophenyl-β-D-glucopyranoside made possible calculation of the pK_a values of catalytic groups which were approx. 7.1 and 4.2. It appears that the very same nucleophilic (carboxyl) group with pK_a 4.2—4.4 participates both in the processes of enzymatic hydrolysis and in the enzyme inactivation by isothiocyanate V. Possibly, this is also true of proton-donating group though its pK_a value in the inactivation is shifted into the acid area (5.6 instead of 7.1). Such an assumption seems probably correct, taking into account that the nitrogen atom of compound V in the enzyme-inhibitor complex being subjected to protonation ought to be at the same place as the oxygen of the substrate's glycosidic bond (Fig. 10).

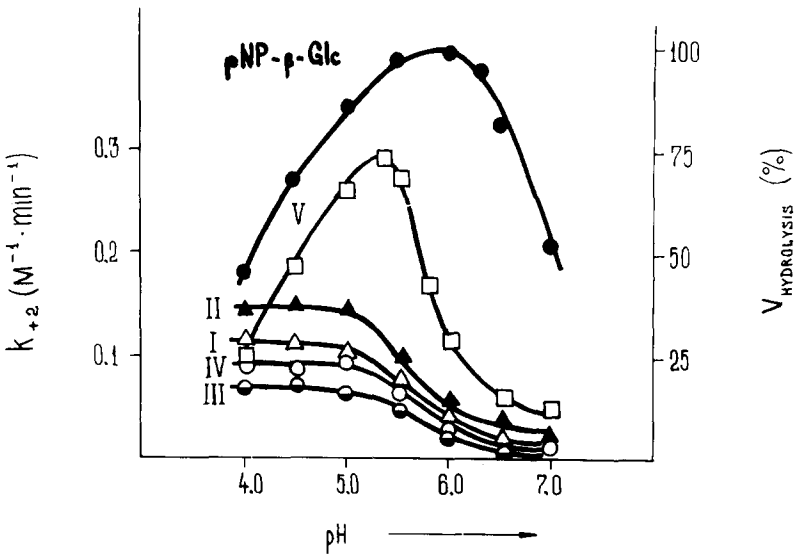


Fig. 9. pH dependence of inactivation rate of component B with irreversible inhibitors I—V at [E] = 10^{-6} M, [I] = $5 \cdot 10^{-2}$ M (left ordinate) and of hydrolysis rate of *p*-nitrophenyl-β-D-glucopyranoside (upper curve) at [E] = 10^{-7} M. [S] = 0.02 M (right ordinate); each point is a result of three measurements.

For epoxides I—IV the pH dependence of the inactivation rate has a sygmoidal character (Fig. 9) and points to the involvement in the process of the proton-donating group of the enzyme with pK_a 5.8—5.9, as is the case for isothiocynate V (p*K* approx. 5.6).

As it appears from Fig. 9 and Table II, there is a slight but distinct difference in the constants of the competitive (K_i) and irreversible (k_{+2}) enzyme inhibition by epoxides I—IV. On the whole epoxypropanes III and IV are somewhat weaker inhibitors than epoxyethanes I and II; possibly, this is due to the removal of the epoxide function from the reaction site within the enzyme-inhibitor complex. In turn, the diastereomeric epoxides of each pair (I, II and III, IV) also differ in their degree of affinity for the active site and the rate at which they inactivate the enzyme. Bearing in mind the significance of the proton-donating group of the enzyme in the process of inactivation (Fig. 9) one may assume that the possible cause of the observed differences lies in the difference of orientation of the epoxide ring oxygen relative to the enzyme proton-donating

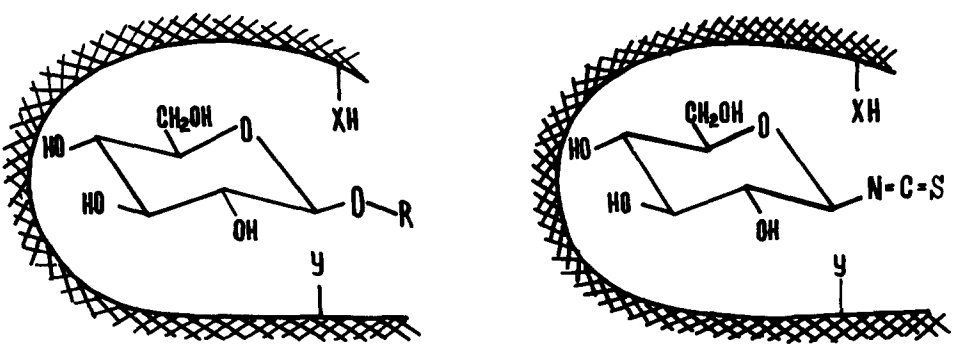


Fig. 10. Enzyme-substrate and enzyme-inhibitor (V) complexes.

group conditioned by the interaction of this ring with the pyranose ring and the protein polypeptide chain.

Of the two diastereomeric galactopyranosylepoxyethanes VI and VII, only isomer VI [15] irreversibly inactivates the enzyme and, as a result, simultaneous suppression of both the glucosidase and galactosidase activity (as inhibs. I—IV) is seen. This correlates with well-established data [23,27,28] which testify that both activities belong to the same active site of the enzyme.

Thus, synthesized compounds I—VI are specific irreversible inhibitors of sweet-almond glucosidase. Our preliminary tests have shown that β-D-galactopyranosylepoxyethane (VII) irreversibly inactivates β-galactosidase from *Curvularia inaequalis*, * which has pH optimum at 4.2. It ought to be assumed that similar inhibitors obtained from monosaccharides other than glucose and galactose may be used for affinity labelling of other glycosidases.

Acknowledgement

The authors are indebted to Dr. A.S. Tikhomirova and Dr. N.A. Zagustina for the specimen of β-galactosidase.

References

1 Baker, B.R. (1967) Design of Active-Site-Directed Irreversible Enzyme Inhibitors, John Wiley and Sons, New York
2 Singer, S.J. (1967) Adv. Protein Chem. 22, 1—54
3 Thomas, E.W., McKelvy, J.F. and Sharon, N. (1969) Nature 222, 485—486
4 Eshdat, Y., McKelvy, J.F. and Sharon, N. (1973) J. Biol. Chem. 248, 5892—5898
5 Legler, G. and Bause, E. (1973) Carbohydr. Res. 28, 45—52
6 Legler, G. (1966) Hoppe-Seyler's Z. Physiol. Chem. 345, 197—214
7 Legler, G. and Hasnain, S.N. (1970) Hoppe-Seyler's Z. Physiol. Chem. 351, 25—31
8 Bause, E. and Legler, G. (1974) Hoppe-Seyler's Z. Physiol. Chem. 355, 438—442
9 Yariv, J., Wilson, K.J., Hildesheim, J. and Blumberg, S. (1971) FEBS Lett. 15, 24—26
10 Naider, F., Bohak, Z. and Yariv, J. (1972) Biochemistry 11, 3202—3211
11 Quaroni, A., Gershon, E. and Semenza, G. (1974) J. Biol. Chem. 249, 6424—6433
12 Thomas, E.W. (1970) Carbohyd. Res. 13, 225—228
13 Shulman, M.L., Shiyan, S.D. and Khorlin, A.Ya. (1974) Carbohydr. Res. 33, 229—235
14 Khorlin, A. Ya., Shiyan, S.D., Lakhtina, O.E. and Shulman, M.L. (1975) Bioorg. Khim. 1, 393—395
15 Shiyan, S.D., Lakhtina, O.E., Shulman, M.L. and Khorlin, A.Ya. (1976) Izv. Akad. Nauk. S.S.S.R., Ser. Khim., in the press
16 Helferich, B. and Kleinschmidt, T. (1965) Hoppe-Seyler's Z. Physiol. Chem. 340, 31—45
17 Kleinschmidt, T. and Horst, J. (1971) Hoppe-Seyler's Z. Physiol. Chem. 352, 603—614
18 Isbell, H.S. and Frash, H.L. (1933) J. Res. Natl. Bur. Stand. 11, 649—654
19 Vasiliev, A.D., Andrianov, V.I., Simonov, V.I., Shiyan, S.D. and Shulman, M.L. (1976) Bioorg. Khim. 2, 601—610
20 Taverna, R.D. and Langdon, R.G. (1973) Biochem. Biophys. Res. Commun. 54, 593—599
21 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
22 Conchie, J. and Levvy, G.A. (1957) Biochem. J. 65, 389—395
23 Heyworth, R. and Walker, P.G. (1962) Biochem. J. 83, 331—335
24 Legler, G. and Witassek, F. (1974) Hoppe-Seyler's Z. Physiol. Chem. 355, 617—625
25 Helferich, B. and Kleinschmidt, T. (1968) Hoppe-Seyler's Z. Physiol. Chem. 349, 25—28
26 Alberty, R.A. and Massey, V. (1954) Biochim. Biophys. Acta 13, 347—353
27 Conchie, J., Gelman, A.L. and Levvy, G.A. (1967) Biochem. J. 103, 609—615
28 Kelemen, M.V. and Whelan, W.J. (1966) Arch. Biochem. Biophys. 117, 423—428
29 Zagustina, N.A. and Tikhomirova, A.S. (1976) Biokhimiya, 41, 1061—1066

* The isolation and characterization of this enzyme are described in ref. 29.