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INHIBITION OF β-GLUCOSIDASES FROM ALMONDS BY CATIONIC AND NEUTRAL β-GLUCOSYL DERIVATIVES

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Summary

The β-glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21) isoenzymes B from sweet and bitter almonds showed considerable differences in their kinetic and inhibition parameters, but both were inhibited much more strongly by basic β-glucosyl derivatives than by their neutral analogs. The additional interaction energy apparently due to the basic character ranged from 18 kJ/mol (4.3 kcal/mol) for β-glucosylamine compared to β-glucose to 28 kJ/mol (6.9 kcal/mol) for *N*-benzyl-β-glucosylamine compared to *N*-β-glucosyl-*p*-toluidine. *N*-β-Glucosylpyridinium ion and *N*-β-glucosylimidazol which both cannot be protonated at the glucosylated nitrogen are very weak inhibitors. β-2-Amino-2-deoxyglucose is bound with half the affinity of β-glucosylamine. The structural requirement for strong inhibition is thus the protonation of the inhibitor at the glucosylated nitrogen. The additional binding energy is assumed to be due to the electrostatic interaction of the inhibitor cation with a carboxylate group in an environment of low polarity. The failure of the pyridinium ion to show this interaction is attributed to the presence of a positively charged group at the active site which acts as proton donor. The pK_a values of β-glucosylamine and its derivatives have been determined and found to be 3.5 units lower than those of the corresponding parent amines. An exception is β-glucosylimidazol (pK_a 5.4) which is protonated on the non-glycosylated nitrogen.

Introduction

From the irreversible inhibition of β-glucosidases (β-D-glucoside glucohydrolase, EC 3.2.1.21) including those from almonds by conduritol B epoxide we deduced the presence of a carboxylate anion at the active site of these enzymes. The carboxylate ion and an acidic group protonating the epoxide oxygen are thought to be involved in the enzymatic hydrolysis of β-glucosides [1]. The presence of a negatively charged group at the active site of many

glycosidases is also indicated by the finding of Lai and Axelrod [2] that these enzymes are inhibited very strongly by glycosylamines corresponding to their sugar specificities. There is, on the other hand, a report by Loeffler et al. [3] that β-glucosidases from sweet almonds might bear a positive charge at the active site. In order to learn more about the groups at the active site and their environment we have investigated the inhibition of β-glucosidases from sweet and bitter almonds by β-glucosylamine and a series of its N-substituted derivatives with different basic character.

Experimental

Substrates and inhibitors were synthesized by published procedures: 4-methylumbelliferyl-β-glucopyranoside [4], *p*-nitrophenyl-β-glucoside [5], β-glucosylamine [6], *N*-bromoacetyl-β-glucosylamine [7], *N*-benzyl-β-glucosylamine [8], *N*-β-glucosylpiperidine [9], *N*-β-glucosylpyridinium bromide [10], *N*-β-glucosyl p-toluidine [11], *N*-β-glucosylimidazol [12], β-glucosyl benzene [13], benzyl-1-thio-β-glucosylpyranoside [14]. β-Glucose was prepared by crystallisation of glucose from pyridine [15], β-2-deoxy-2-amino glucose by treating the hydrochloride with dimethylamine in ethanol [16]. All glucose derivatives were of D-configuration. Melting points and specific rotations agreed satisfactorily with the values given in the references cited.

Enzymes. β-Glucosidases A and B from bitter almonds were obtained by the procedure described for sweet almonds [17]. Defatted almond meal (Cesar and Loretz, D-4010 Hilden) was used as starting material. In order to prevent excessive release of hydrocyanic acid it was first extracted with 6 volumes of acetone/water, 3 : 2 (v/v), to remove amygdalin, then with acetone, dried and processed as in ref. 17.

β-Glucosidase B from sweet almonds was isolated from a commercial enzyme preparation (β-glucosidase from Sigma Chem. Co., St. Louis, U.S.A., lot No. 95C-0229), starting with the first acetone precipitation of ref. 17. Specific activities are expressed as units/mg protein determined with 20 mM *p*-nitrophenyl-β-D-glucoside at pH 5.0 and 35°C unless specified otherwise.

Protein concentrations were determined by ultraviolet absorption at 280 nm with $A_{280}^{1\,mg/ml} = 1.88$ and 1.82 for β-glucosidases A and B respectively [17].

Rate measurements and inhibition studies. Substrate solutions were prepared in the following buffers (all 50 mM): pH 4.0, 5.0, and 6.0 sodium acetate/hydrochloric acid; 6.5, 7.0, and 8.0 disodium hydrogen phosphate/hydrochoric acid.

Rate determinations with *p*-nitrophenyl-β-D-glucoside were done in the concentration range 1—20 mM at 25°C.

The rate with 4-methylumbelliferyl-β-D-glucoside (1 mM) was determined with a Zeiss PMQ II spectrophotometer with ZFM 4 fluorescence attachment by following the time course of fluorescence at 440 nm (excitation at 365 nm) as recorded by a Servogor S recorder (Metrawatt). The fluorescence was standardized by adjusting the signal from a $5 \cdot 10^{-5}$ M solution of 4-methylumbelliferone in the assay buffer to 100%. Release of product was calculated from the recorded fluorescence intensity and the concentration of standard. The temperature was held at 25°C by a thermostatted cuvette holder and the use of

thermally equilibrated substrate solutions. Product release was linear up to about 50% fluorescence except for assays at pH 7.0 or above. At these pH values ionisation of 4-methylumbelliferone to its much stronger fluorescing, but radiation-sensitive anion became noticeable. Radiation damage caused deviations from linearity to occur earlier but initial rates could still be measured with sufficient accuracy.

For the inhibition studies inhibitor solution was added with a microliter syringe to the substrate solution that had been preincubated at the desired temperature. Enzyme solution was added immediately afterwards. To minimize changes in inhibitor concentration due to conversion to the α-anomer or hydrolysis the solutions of the glucosylamines were used immediately after dissolution. In some cases stock solutions with 100 times the final concentration were prepared in acetonitril. If a preliminary experiment had shown a pH shift of more than 0.1 units to occur when substrate and inhibitor solution were mixed, an amount of hydrochloric acid equivalent to the inhibitor was added to the substrate before use.

N-benzyl-β-glucosylamine could not be used at pH 5 or below due to the acid-catalyzed decomposition of the inhibitor, which caused a considerable rate increase after 1—2 min.

V and K_m were obtained graphically from Lineweaver-Burk plots. Inhibition constants for competitive inhibition K_i^c were calculated from the slope in the presence of inhibitor (slope (I)) related to the slope in its absence (slope (0)). Constants for anticompetitive inhibition were calculated similarly from the respective ordinate intercepts [18]:

$$K_i^c = \frac{[I]}{\frac{\text{slope (I)}}{\text{slope (0)}} - 1} \qquad K_i^a = \frac{[I]}{\frac{\text{intercept (I)}}{\text{intercept (0)}} - 1}$$

Values for V were reproducible within ± 5%, K_m and K_i values within ± 15%.

Acid dissociation constants of protonated glucosylamines. Due to the lability of most glycosylamines in aqueous solution a potentiometric titration is not possible. Dissociation constants were therefore determined by dissolving an accurately weighed amount of glucosylamine in one-half of the calculated amount of 0.1 M hydrochloric acid (0.5 M in the case of *N*-β-glucosyl-*p*-toluidine) and measuring the pH immediately after dissolution with a Metrom E 500 digital pH meter. This pH was considered to be equal to the apparent pK_a. Duplicate determinations agreed within 0.03 pH units.

Results and Discussion

Acid dissociation constants of glucosylamines

Because we expected an interaction of the presumed carboxylate group at the active site with the cationic form of the inhibitors, dissociation constants were determined to calculate the concentration of this species and to obtain a relative scale of basicity. The results are summarized in Table I together with pK_a values for the parent amines taken from the literature. Except for the imidazol derivative, where protonation is expected to take place on the non-

TABLE I

DISSOCIATION CONSTANTS OF PROTONATED β-GLUCOSYLAMINES

Parent amine	pK_a	pK_a of β-glucosyl derivative
Ammonia	9.25	5.6
Benzylamine	9.33	5.3
Imidazol	6.95	5.4
Piperidine	11.12	6.1
p-Toluidine	5.08	1.5

glucosylated nitrogen [19], the pK_a shifts correspond to those observed with amino acids after conversion to their bis-hydroxymethyl derivatives [20]. Binding of the glucosylamine to the active site will, of course, alter the dissociation constant, but one can expect that the relative order of basicity will remain essentially the same.

With the non-aromatic glucosylamines the pH changed to lower values immediately after dissolution in the acid and started to rise after 5—10 min. Final pH values corresponded to those expected for complete hydrolysis to neutral carbohydrate and free amine. The initial drop in pH is attributed to an isomerisation to the α-glucosylamine, which is expected to have a lower pK_a due to the reverse anomeric effect which stabilises the cation more in the β-anomer [21]. This isomerisation is also assumed to be the cause for the observed change in optical rotation [22].

β-Glucosidases A and B from sweet and bitter almonds

The two principal β-glucosidase isoenzymes from sweet almonds were designated A and B by Helferich [23] and are characterized by their different elution behaviour on DEAE-cellulose, different molecular weight, and specific activities [17]. For technical reasons we first used bitter almond meal as starting material, where β-glucosidases were separated into two analogous fractions on DEAE-cellulose under the same conditions. β-Glucosidase A appeared to be homogeneous by ion-exchange chromatography, gel chromatography and SDS-gel electrophoresis. In contrast to a β-glucosidase A from a different batch of almond meal [24] it was separated, however, into four subfractions by isoelectric focussing (isoelectric point 5.7 to 6.7) which all had very similar specific activities (≈ 110 units/mg) [25].

The relative activity of these enzymes against *p*-nitrophenyl-β-glucoside (20 mM) (v_{PNPG}) and 4-methylumbelliferyl-β-glucoside (1 mM) ($v_{4\text{-MUG}}$) (both at pH 5.0 and 25°C) was $v_{PNPG}/v_{4\text{-MUG}} = 0.28$. This ratio was found to differ widely for β-glucosidases A and B and we propose to use it for the characterisation of almond glucosidase of different origin. The value found for A from sweet almonds was $v_{PNPG}/v_{4\text{-MUG}} = 0.10$. β-Glucosidase B was separated on DEAE-cellulose into at least three overlapping subfractions with $v_{PNPG}/v_{4\text{-MUG}} =$ 4.5, 10 and 20. Complete separations of these subfractions and of the flavoprotein hydroxynitril-lyase overlapping with the last (main) fraction was possible only with great losses. Maximal specific activity was 320 U/mg (610 U/mg had been found for B from sweet almonds [17]). The data given in Table II

TABLE II

INHIBITION OF β-GLUCOSIDASE B FROM BITTER ALMONDS (pH 6.0 AND 35°C) AND SWEET ALMONDS (pH 6.0 AND 25°C). VALUES FOR K_i ARE BASED ON TOTAL INHIBITOR CONCENTRATION

	Bitter almonds		Sweet almonds			
	K_i^c	$\Delta\Delta G$ (kJ/mol) *	K_i^c (mM)	K_i^a/K_i^c	$\Delta\Delta G$ (kJ/mol) *	
β-Glucose	450		80	30		
β-2-Amino-2-deoxyglucose	—	—	6	30	6	
β-Glucosylamine	2.0	13	0.31	8	13.5	
N-Bromoacetyl-β-glucosylamine	60	3.6	—	—	—	
D-Glucono-δ-lactone	—	—	0.051	3	18	
β-Glucosylbenzene	—	—	40	9		
β-Glucosylpyridinium ion	150	<0 **	46	1.3	<0	***
N-β-Glucosylpiperidine	0.0035 0.0015 ††	23.5 **	—	—	—	
N-β-Glucosyl-*p*-toluidine	54		—	—	—	
N-β-Glucosylimidazol	500	<0 **	—	—	—	
Benzyl-1-thio-β-glucoside	—	—	1.4	2.0		
N-Benzyl-β-glucosylamine	0.00026	28 **	0.00032	1.6	20.5 †	
Benzylamine	1.3		—	—	—	

* Additional interaction energy calculated from $\Delta\Delta G = RT \ln K_i^c/K_i^c$ (ref); K_i^c (ref) is the inhibition constant for β-glucose except where indicated otherwise.

** *N*-β-Glucosyl-*p*-toluidine as reference compound.

*** β-Glucosylbenzene as reference compound.

† Benzyl-1-thio-β-glucoside as reference compound.

†† Calculated from the concentration of free base if the cation is assumed to be non-inhibiting.

were measured with the last fraction; in still contained about 5% flavoprotein.

The difficulties in obtaining a pure enzyme prompted us to use commercial almond glucosidases from sweet almonds as starting material. A preparation from Calbiochem turned out to contain almost exclusively a single component with the properties of β-glucosidases B that could be enriched from 6.2 to 520 U/mg protein and that showed $v_{PNPG}/v_{4\text{-}MUG} = 17$. Differences between B from sweet and bitter almonds were: different pH optima (6.2 vs. 4.8 with *p*-nitrophenyl-β-D-glucoside) and a different pH dependency of K_m.

Inhibition studies

Constants for competitive inhibition found with β-glucosidase B from sweet and bitter almonds are given in Table II. With both enzymes an enhancement of binding by the basic and hydrophobic properties of the inhibitors is observed in spite of considerable differences in the absolute values. Measurements at 35°C showed a purely competitive inhibition and no substrate inhibition with both glucosidases. The enzyme from sweet almonds was also investigated at 25°C where mixed competitive/non-competitive inhibition and substrate inhibition were observed. Some exploratory measurements with β-glucosidase A from bitter almonds showed a similar influence of the basic and hydrophobic properties of the inhibitor and a similar temperature effect on the mode of inhibition.

The following information can be obtained from a detailed consideration of the inhibition data: a sufficiently basic group at C-1 of the glucose residue

contributes an additional interaction energy compared to the non-basic structural analog that ranges from −13.5 kJ/mol (−3.3 kcal/mol) without nitrogen substituent to −20.5 kJ/mol (−4.9 kcal/mol) if we compare *N*-benzyl-β-glucosylamine with benzyl-1-thio-β-glucoside or to −31 kJ/mol (−7.4 kcal/mol) if we compare it with *N*-β-glucosyl-*p*-toluidine. The standard free energies of binding β-glucose, benzylamine, and *N*-benzyl-β-glucosylamine to the bitter almond enzyme as calculated from K_i^c are −2.1, −17, and −38.5 kJ/mol, respectively. The additional energy of −19.4 kJ/mol found with *N*-benzyl-β-glucosylamine in comparison to the sum of β-glucose plus benzylamine can be interpreted either as an entropic contribution (the "anchor principle" [26]) or as an induced fit effect of the glucosyl residue that permits a closer approximation of the protonated nitrogen to a negatively charged group at the active site.

The inhibition constants found for β-glucosylbenzene, β-glucosylpyridinium ion, and β-glucosylpiperidine deserve special comment. The K_i values for the first two confirm the observation of Sinnott's group [3] that the positively charged inhibitor is bound less firmly than its neutral analog. That this weak interaction is not due to branching at the substituent at C-1 of the glucose residue is shown by the additional binding energy of −27 kJ/mol (−6.4 kcal/mol) found with *N*-β-glucosylpiperidine in comparison with the pyridinium derivative.

A possible explanation for this detrimental influence of a permanent positive charge in the inhibitor could be the presence of a protonated base (BH^+)-carboxylate ion pair at the active site. Proton transfer from BH^+ would form the inhibitor cation, provided it can accept the proton. The ion pair would now consist of the protonated inhibitor and the carboxylate group. This proton transfer is not possible with the pyridinium derivative; electrostatic attraction by the carboxylate group and repulsion by BH^+ would cancel to an extent depending on the distances between the three charged groups. Positive differences in the binding energy between benzene and pyridinium derivative might also be due to differences in the energy of desolvation which is expected to be higher for the ionic inhibitor.

An interpretation of pH effects (Fig. 1) is possible only with great reservations because several pH-dependent processes will overlap: ionisation of BH^+, if this is a protonated imisazol, of free and bound inhibitor, and, on the acid side, of the carboxylate group. In addition, one has to take account of a pH-dependent conformation change around pH 7 that was indicated for β-glucosidase B from sweet almonds by changes in tryptophan fluorescence [27]. The most pronounced feature is the difference between the pyridinium derivative and the other inhibitors. The plateau above pH 7 could be explained by an increasing deprotonation of BH^+, thus reducing the repulsive interaction. This deprotonation would, of course, prevent the proton transfer to the basic inhibitors, thus causing a weakening of the inhibition.

The following tentative conclusions can be drawn from the results presented in this paper: the β-glucosidases from sweet and bitter almonds have a carboxylate anion at the active site which stabilizes the binding of basic inhibitors by an additional interaction energy compared to their non-basic analogs of 14 to approx. 25 kJ/mol. This stabilizing effect requires a low dielectric constant in

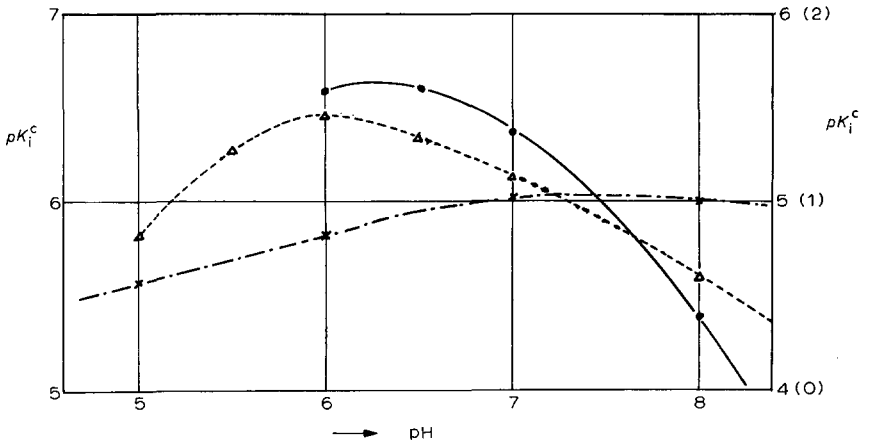

Fig. 1. pH dependence of the inhibition of β-glucosidase B from bitter almonds at 35°C by *N*-benzyl-β-glucosylamine (●———●; left ordinate); *N*-β-glucosyl piperdine (△- - - - -△; right ordinate), and β-glucosyl pyridinium ion (X- - - - - -X, right ordinate, figures in brackets).

the environment of this group. If we assume that this additional energy is due to Coulomb forces alone a value of $\epsilon \approx 15$ may prevail (the Coulomb energy of an ion pair separated by 0.4 nm amounts to 23 kJ/mol with $\epsilon = 15$). The relatively weak inhibition by 2-amino-2-deoxy-β-glucose compared to β-glucosylamine shows that the interaction between carboxylate and the cationic group of the inhibitor depends critically on the relative positions of the two groups. This points to a fairly rigid structure of the EI complex.

Another reason for the enhanced binding of the basic glucosyl derivatives could be their greater ability to form hydrogen bonds with suitably oriented complementary groups. If water is excluded from the active site this might also give a substantial contribution to binding. It seems doubtful, however, that differences in the ability to form hydrogen bonds between analogous basic and non-basic inhibitors will produce effects as large as have been observed here.

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