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Evidence that β-Galactosidase of *Sulfolobus solfataricus* Is Only One of Several Activities of a Thermostable β-D-Glycosidase

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A survey of *Sulfolobus* isolates showed all to contain thermostable enzyme activities hydrolyzing various glycosidic compounds. Of those not previously reported, the β-glucosidase activity of *Sulfolobus solfataricus* isolate P2 was chosen for further study and found to have the same kinetics of inactivation, apparent molecular weight, and many (though not all) other biochemical properties of the β-galactosidase also present in this strain. The two activities copurified approximately 850-fold to apparent homogeneity. The enzyme, whose subunit M_r was estimated to be 60,000 to 65,000 by gel permeation chromatography of the active enzyme and 70,000 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the denatured form, hydrolyzed a variety of low-molecular-weight, β-linked glycosides and could account for most of the corresponding activities found in crude extract. Kinetic analyses indicated that chromogenic β-D-galactosides and β-D-glucosides are hydrolyzed at a common active site and that β-glucosides and β-fucosides represent the preferred substrates. The liberation of aglycone from aryl β-D-glucosides was stimulated by alcohols in a manner suggesting specific interaction between alcohol and enzyme.

Current efforts to isolate and characterize enzymes from members of the sulfur-metabolizing, thermophilic branch of the archaebacteria are motivated by their extremely thermostable nature as well as by their usefulness for molecular phylogeny. The structural features that enable thermostable enzymes to remain active at high temperatures are diverse and incompletely understood, but they tend to stabilize the enzyme toward other denaturing treatments as well (14). Thus, enzymes from extremely thermophilic archaebacteria, some of which grow above 100°C (23), warrant evaluation as both model systems and possible catalysts in industrial processes.

The genus *Sulfolobus* has provided a particularly favorable context for experimental biochemistry of this evolutionary lineage, due in part to its ease of cultivation compared with other extremely thermophilic archaebacteria. One of the enzymes of *Sulfolobus* under active study is the β-galactosidase from *Sulfolobus solfataricus*, whose thermostability and tolerance of organic solvents have been evaluated for the purpose of hydrolyzing lactose in dairy products (3). More recently, the enzyme has been purified and characterized in greater detail (19), and its gene has been cloned (5). The availability of sophisticated biochemical and genetic tools for analysis of β-galactosidases thus makes this enzyme potentially useful as a model of structure-function relationships in extremely thermostable enzymes. Results of the present study demonstrate, however, that certain basic properties of this enzyme have not been recognized, including its preference for substrates other than β-D-galactosides.

(Portions of this work have been presented previously [11].)

MATERIALS AND METHODS

Bacterial strains and growth conditions. The clonally purified cultures of *Sulfolobus* isolates used in this study and heterotrophic growth conditions have been described previously (10). The cell extracts of *S. solfataricus* P2 were prepared by resuspension of cells in about 0.7 ml of 50 mM sodium phosphate (pH 6.8), followed by 20- to 60-s sonication and removal of unbroken cells by centrifugation (10 min, 12,000 × *g*) or by sodium sarcosyl extraction, performed in the course of purifying S-layer cell walls as described previously (10); the detergent extract was dialyzed versus 50 mM sodium phosphate (pH 6.5), stored at −20°C with 40% (vol/vol) glycerol added, and redialyzed before use to remove glycerol.

Glycosidase assays. Unless otherwise indicated, glycosidase activities were assayed by determining the formation of free 4-nitrophenol from the corresponding glycoside. The standard assay mixture consisted of 100 mM KCl, 100 mM sodium citrate (pH 4.9), 20 mM dithiothreitol (DTT), 1.6 mM substrate, and enzyme in a total volume of 0.20 ml. The reaction was started by mixing the components at 20°C followed by rapid equilibration (13-mm thin-walled glass tubes vigorously agitated) with a 77°C water bath; it was stopped by similarly chilling the tubes in ice water followed by addition of 0.15 ml of 1 M Na_2CO_3.

Hydrolysis of nonchromogenic substrates was calculated on the basis of D-glucose liberated. Aliquots of an assay mixture (as above, except 20 mM DTT and chromogenic substrate were replaced by 5 mg of nonchromogenic substrate per ml) were withdrawn from 77°C incubation at regular intervals, chilled, and assayed with glucose oxidase reagent essentially as described by Dekker (6), except that oxidation of *p*-phenylenediamine was assayed by optical density at 405 nm. Simultaneous liberation of D-glucose and 2-hydroxymethylphenol from salicin was assayed as follows: aliquots from the assay mixture were diluted 1:4 in 1 M Na_2CO_3 at 20°C. The released aglycone was assayed by A_{292}, based on an empirically determined molar extinction coefficient difference of 3,400 per cm. Free glucose was assayed as reducing sugar by the formation of a highly colored Fe^{2+} complex (1). To assess the stability of glycosidase activities to various denaturing conditions, extract was diluted in citrate buffer; aliquots (20 μl) were withdrawn at

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regular intervals, and the residual activity was assayed under standard conditions in a final volume of 0.20 ml.

Enzyme purification. All steps were performed at 20°C except when indicated.

(i) Ammonium sulfate fractionation. Saturated $(NH_4)_2SO_4$ (0.55 volume) was added dropwise to 1.00 volume of crude extract. After 1 h the precipitate was collected by centrifugation and discarded. To the resulting volume of supernatant was added an additional 0.5 volume of saturated $(NH_4)_2SO_4$; after 1 h the precipitate was recovered by centrifugation, suspended in a small volume of 20 mM sodium citrate (pH 5.2), and dialyzed overnight against the same buffer.

(ii) Acetone fractionation. To the resulting dialysate was added 0.8 volume of acetone. The mixture was capped and incubated at 77°C for 15 min, followed by cooling and storage at 4°C for 1 to 2 h. The resulting precipitate was removed by centrifugation and discarded; the supernatant was concentrated two- to threefold by evaporation under a stream of N_2 or air.

(iii) Gel permeation chromatography. To the resulting concentrate was added sodium dodecyl sulfate (SDS), glycerol, and DTT to final concentrations of 0.1%, 5% (vol/vol), and 20 mM, respectively. The sample was heated for 10 min at 75 to 85°C and applied to a column (1 by 30 cm) of Bio-Gel P150 (Bio-Rad) equilibrated in 50 mM sodium phosphate (pH 6.8)–0.1% SDS. Fractions (150 μl) eluted with the same buffer were assayed; the single peak of activity, which eluted after the void volume, was retrieved and stored at 4°C.

(iv) Affinity chromatography. The gel permeation eluate was passed over a small column (0.8-ml bed volume) of agarose beads (Sigma Chemical Co.) derivatized with *p*-aminobenzyl-1-thio-β-D-galactopyranoside groups; the column was washed extensively with the buffer described above and with buffer containing 0.5 M NaCl. Bound enzyme was recovered in the first two to three fractions (0.15 ml each) of 10 mM sodium borate (pH 9.5 [22]), the pH was adjusted to 5 with sodium citrate buffer, and storage was at 4°C. No attempts were made to optimize the storage conditions, and some activity loss (about 50% after 6 weeks) was observed.

Unless otherwise indicated, SDS-polyacrylamide gel electrophoresis (PAGE) used the discontinuous system of Laemmli (15); continuous SDS-PAGE was performed in a miniature format (1.5 by 60 by 80 mm) in 0.1% SDS and 0.10 M each glycine and diethanolamine, pH 9.7. In both cases samples were boiled for 1 to 2 min in the corresponding gel buffer containing 20 mM DTT immediately before electrophoresis. Except when indicated, protein concentrations were determined by small-scale biuret assays (16); the pH was measured at 20°C with a glass electrode.

RESULTS

Glycosidase activities in *Sulfolobus* isolates. A preliminary survey of *S. acidocaldarius* DSM 639 and C, *S. solfataricus* P2 and MT4, and *S. shibatae* B12 (12) showed all to contain thermostable enzyme activities hydrolyzing several glycosidic linkages. All five strains contained β-galactosidase (optimal pH of approximately 4.9) and β-glucosidase (optimal pH of approximately 5.5), although the specific activities in *S. acidocaldarius* cells were typically 5 to 10% those of the other strains. Significant α-galactosidase activity was observed only in strains P2 and MT4, and α-glucosidase was seen only in strains P2 and B12. Of the four glycosidase activities found in *S. solfataricus* P2, only the β-galactosidase had been reported previously (3); the source used for that study, *S. solfataricus* MT4, is very closely related to strain P2 by the criteria of genomic homology (12) and physiological properties (10), indicating that the corresponding enzymes should be essentially the same.

TABLE 1. Stability of β-glycosidase activities

Treatment	Observed half-life (min)	
	β-Glucosidase	β-Galactosidase
pH 5.2[a]		
77°C	>120	>120
87°C	>120	>120
97°C	13	13
77°C + 3% SDS	130[b]	120[b]
77°C + 7 M urea	65[b]	60[b]
77°C[c]		
pH 2.5	2	2
pH 3.6	70	70
pH 5.5	>300	>300

[a] Enzyme stability (crude extract) in 40 mM sodium citrate–5 mM DTT was monitored as described in Materials and Methods, up to a maximum of 90 min.
[b] Calculated from residual activity at 60 min, assuming first-order decay.
[c] Stability was determined in 50 mM Tris-citrate–5 mM DTT.

The most abundant of the previously unreported activities of strain P2, the β-glucosidase, was further characterized in crude extracts, using the β-galactosidase of this strain as an internal reference for comparison. The most notable difference between the two activities was stimulation of β-glucoside hydrolysis by alcohols. Ethanol (10%, vol/vol), for example, doubled the apparent β-glucosidase activity without affecting β-galactoside hydrolysis. This twofold stimulation was observed over the temperature range 66 to 97°C and was not accompanied by destabilization of either activity (data not shown). Stimulation was specific to alcohols, occurring with ethanol, 2-propanol, 1-butanol, 2-butanol, or glycerol but not with other solvents of comparable polarity, i.e., acetone, acetonitrile, dimethyl sulfoxide, dimethyl formamide, ethyl acetate, or tetrahydrofuran, and was not observed with the β-galactosidase activity.

In other respects, however, the two major glycosidase activities of *S. solfataricus* P2 proved very similar. Both were stimulated 30 to 40% by 0.1 M KCl and by 20 mM DTT, both remained active and soluble in high concentrations (up to 50%, vol/vol) of ethanol, 2-propanol, acetone, or acetonitrile at 77°C despite considerable precipitation of other proteins, and both were inactivated at the same rate by a variety of denaturing treatments (Table 1).

The observed stability of both activities toward SDS, even at high temperature, was exploited in an attempt to differentiate the two on the basis of electrophoretic mobility of the active enzyme in SDS-PAGE. Crude extract was treated with 0.1% SDS at 80°C and electrophoresed in the system of Weber and Osborn (25); 2-mm slices of the resulting gel were eluted and the eluates were assayed. The β-glucosidase and β-galactosidase activities were found to comigrate precisely, yielding a constant ratio of activities across the peak. This peak migrated well behind the large S-layer glycoprotein subunit of strain P2 (provided as an M_r standard), which corresponded to an apparent M_r of >200,000 (data not shown). This provided no evidence of the true M_r, however, since an active enzyme is expected neither to have bound significant amounts of SDS nor to have become unfolded.

TABLE 2. Copurification of β-glucosidase and β-galactosidase activities[a]

Purification step	Protein		β-Galactosidase activity				β-Glucosidase activity			
	mg	mg/ml	U	U/mg of protein	% Recovery	Purification factor	U	U/mg of protein	% Recovery	Purification factor
Crude extract	336	19	49	0.15	100	1	26	0.077	100	1
35 to 65% $(NH_4)_2SO_4$	57	11	32	0.56	65	3.8	16	0.28	62	3.6
77°C acetone precipitation	7.5	2.5	25	3.3	51	23	14	1.9	54	25
Molecular sieve column	0.35	0.12	11	31	22	213	6.2	18	24	230
Affinity column	0.07[b]	0.2[b]	8.7	124[b]	18	830[b]	4.8	68[b]	18	880[b]

[a] See Materials and Methods for experimental details; data were averaged from two similar trials.
[b] Estimated from the UV spectrum of the sample, assuming 31 tyrosine residues per 61 kD of protein (19) and a molar extinction coefficient of 1,500 per cm at 280 nm for each tyrosine residue.

Gel permeation chromatography under similar conditions (see Methods and Materials) consistently yielded a single symmetric peak of activity eluting slightly behind bovine serum albumin, which indicated an M_r of 60,000 to 65,000; in all trials, the ratio of the two activities was constant across this peak (data not shown). This result indicated that comigration of the two activities in SDS-PAGE was not due to an SDS-stable association between the β-galactosidase and a distinct β-glucosidase. In fact, the brief SDS heat treatment appeared to have dissociated the β-galactosidase into its subunits (19), which retained activity.

Purification. The most straightforward explanation for the above data was that the β-galactosidase and β-glucosidase activities reside on the same enzyme. This was confirmed by purifying a small quantity of the enzyme. The scheme used (see Materials and Methods) took advantage of the above observation that both activities remain soluble and active after heating to 77°C in 45% (vol/vol) acetone, whereas the bulk of *Sulfolobus* cell protein precipitates. This treatment preceded by ammonium sulfate fractionation and followed by the above-described gel permeation chromatography achieved 200- to 300-fold purification of both activities (Table 2), which correlated with a low-mobility band on SDS-polyacrylamide gels (Fig. 1, left panel). After a final affinity chromatography step (Table 2), the preparations yielded only this slowly migrating band on SDS-polyacrylamide gels (Fig. 1, right panel). This low-mobility band was consistent with the electrophoretic mobility of enzymatic activity and appeared to represent an incompletely denatured, if not native, protein; severely denaturing treatment before electrophoresis (97°C for 15 min in 10 M urea–0.8% SDS) converted it to a form migrating at an apparent M_r of about 70,000 (Fig. 1, cf. lanes F and G). Allowing for the different gel systems used, these results are in reasonable agreement with the recent purification of β-galactosidase from *S. solfataricus* MT4 (19).

Substrate specificity. Several potential substrates and substrate analogs were tested for the ability to inhibit 4-nitrophenyl glycoside hydrolysis, and few were found to be effective (Table 3). The more effective compounds included derivatives of both D-galactose and D-glucose and preferentially inhibited the β-galactosidase activity (Table 3), suggesting overlap or steric similarity of the corresponding active sites. To confirm a common catalytic site preferring β-D-glucosides over β-D-galactosides, rates of 4-nitrophenol production were compared by using each chromogenic substrate and an equimolar mixture of the two (4). The qualitative result was a pronounced inhibition of β-galactoside hydrolysis by the β-glucoside; the approximate V_{max} values observed in this experiment (230 U of enzyme per mg for the β-galactoside alone, 70 U/mg for the β-glucoside alone, and 89 U/mg for the equimolar mixture) gave good agreement with a V_{max} of 85 U/mg for the mixture calculated by using the kinetic constants (see below) and the relationship $K_A(V_C$

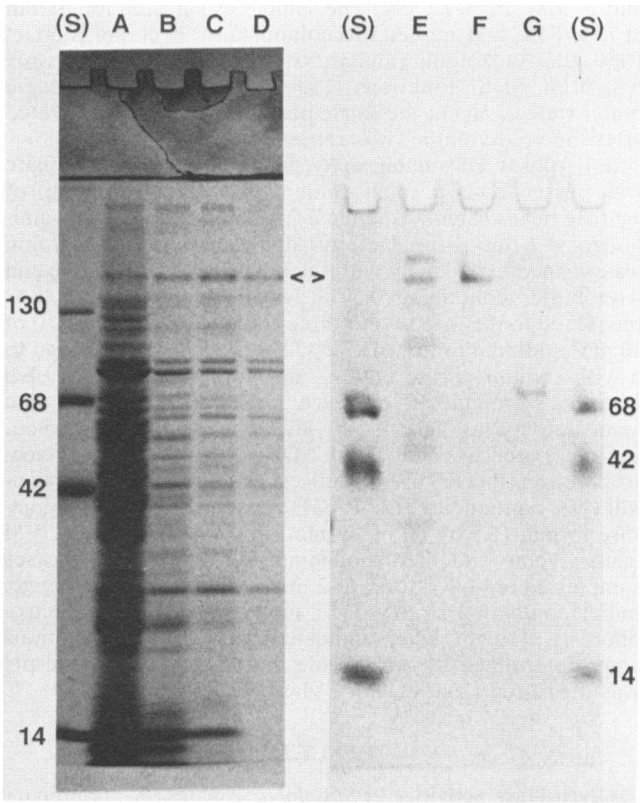

FIG. 1. SDS-PAGE of purification fractions. Left panel shows 7.5 to 15% polyacrylamide gradient gel; each lane received the equivalent of 1.3 U of β-galactosidase activity. Right panel shows 10% polyacrylamide continuous gel (see Materials and Methods); each sample contained approximately 0.3 U of β-galactosidase. Samples were prepared through the indicated purification step, as described in Materials and Methods: lane A, ammonium sulfate fractionation; lane B, acetone precipitation; lane C, fraction unadsorbed to DEAE-cellulose (not used); lanes D and E, gel permeation chromatography; lane G, as lane F except sample was boiled for an additional 10 min with additional 0.6% SDS–10 M urea; lanes S, molecular weight standards (*E. coli* β-galactosidase, bovine serum albumin, ovalbumin, and lysozyme; corresponding molecular mass, in kilodaltons, is indicated along outside margins).

TABLE 3. Effectiveness of substrate analogs as inhibitors of β-glycosidase[a]

Addition[b]	Relative rate of hydrolysis[c]	
	β-Galactoside	β-Glucoside
None	100	100
D-Galactose	79	106
D-Glucose	63	114
Lactose	59	82
D-Fucose	42	77
D-Arabinose	36	80
D-Cellobiose	21	75
Salicin	3	8
1,4-D-Galactonolactone	2	10
1,5-D-Gluconolactone	<1	<1

[a] Highly purified enzyme (purified through gel permeation step; approximately 40 U of β-galactosidase per mg of protein) assayed with 1.6 mM concentration of the indicated 4-nitrophenyl-β-glycoside under standard conditions.
[b] All additives present at 0.10 m final concentration. Under these conditions, sucrose, D-mannose, isopropyl-thio-β-D-galactopyranoside, L-fucose, and L-arabinose failed to inhibit either activity significantly.
[c] 100% values correspond to 2.5 (β-galactosidase) or 3.2 (β-glucosidase) nmol of 4-nitrophenol released per min.

$- V_B) = K_B(V_A - V_C)$, which describes two substrates competing for the same active site (4).

As Table 4 shows, most β-glycosidase activities observed in crude extracts could be attributed to the enzyme purified according to Table 2, whereas a few activities, including α-glucosidase and possibly, hydrolysis of carboxymethyl cellulose and 1-*O*-methyl-β-D-glucoside, did not purify with the β-glycosidase. Standard kinetic analysis showed that, of the six chromogenic substrates hydrolyzed at significant rates by purified enzyme (Table 4), the α-L-arabinoside, β-D-xyloside, and β-D-2-deoxy-2-acetamidogalactoside were relatively poor, characterized by low V_{max} and high K_m values (data not shown). For the remaining chromogenic substrates, the ratio of V_{max}/K_m was found to increase in the order β-D-galactoside < β-D-fucoside < β-D-glucoside (Table 5), showing that the *S. solfataricus* enzyme is properly classified as a β-glucosidase, or perhaps a β-gluco/fucosidase, rather than as a β-galactosidase.

TABLE 5. Kinetic constants for *S. solfataricus* β-glycosidase[a]

Substrate[b]	V_{max} (U/mg of enzyme) ± SE	K_m (μM) ± SE	Relative catalytic efficiency (liters/min/mg of enzyme) ± SE[c]
β-Galactoside	210 ± 71	770 ± 330	0.27 ± 0.02
β-Fucoside	83 ± 23	130 ± 55	0.66 ± 0.09
β-Glucoside	77 ± 18	95 ± 60	0.80 ± 0.30

[a] Constants and standard error were calculated by linear regression of double-reciprocal plots (Sigmaplot program; Jandel Scientific); uncertainty of V_{max} arising from protein determination was not included.
[b] Substrates were the corresponding 4-nitrophenyl-D-pyranosides. Hydrolysis was assayed under standard conditions at 8 to 10 substrate concentrations ranging from 0.03 to 10.0 mM for times ranging from 3 to 12 min; four to six independent rate measurements were made at each substrate concentration. It should be noted that recent results (21) indicate activation by high concentrations of β-galactoside of the enzyme purified from strain MT4; such effects were limited, but not completely eliminated, under the conditions shown above.
[c] Ratio of V_{max} to K_m.

Specificity of stimulation by alcohols. A homologous series of alcohols was tested with purified enzyme to define better the apparent enhancement of enzymatic β-glucoside hydrolysis by alcohols. The relative enhancement of 4-nitrophenol liberation was found to be a complex function of (i) alcohol concentration, (ii) hydrocarbon chain length, and (iii) steric configuration (Fig. 2). The role of each can be summarized as follows.

(i) Each alcohol exerted a "dual" effect, stimulating 4-nitrophenol release at relatively low concentration but inhibiting it at high concentration; this resulted in a well-defined concentration optimum (9).

(ii) The apparent effectiveness of the alcohol for both stimulation and inhibition increased dramatically with length of the hydrocarbon chain. As a result, the concentration optimum for each subsequent member of the homologous

TABLE 4. Comparison of substrate hydrolysis by crude extract and by purified enzyme

Substrate[a]	Hydrolysis			
	Observed rate (nmol/min/μl of enzyme prepn)[b]		Relative rate	
	Crude extract	Pure enzyme	Crude extract	Pure enzyme
4-Nitrophenyl-β-D-galactoside	3.4	3.7	100	100
4-Nitrophenyl-β-D-fucoside	2.8	2.7	81	73
4-Nitrophenyl-β-D-glucoside	1.8	1.9	52	52
4-Nitrophenyl-α-L-arabinoside	0.52	0.52	15	14
4-Nitrophenyl-β-D-xyloside	0.21	0.13	6.1	3.6
4-Nitrophenyl-α-D-glucoside	0.20	0.02	5.8	<0.1
4-Nitrophenyl-β-D-2-deoxy-2-acetamido galactoside	0.16	0.13	4.7	3.6
Salicin	0.23	0.14	6.7	3.8
Cellobiose	0.21	0.12	6.1	3.3
Lactose	0.21	0.12	6.1	3.3
Sucrose	0.06	0.01	1.7	<0.1
1-*O*-Methyl-β-D-glucoside	0.06	0.01	1.7	<0.1
Carboxymethyl cellulose	0.04	<0.01	1.1	<0.1
Starch	0.03	<0.01	0.8	<0.1

[a] All substrates were the pyranoside form; hydrolysis was assayed under standard conditions by visible absorption of the nitrophenylate ion or by oxidation of released D-glucose (see Materials and Methods). Similar tests indicated negligible hydrolysis of 4-nitrophenyl-β-D-2-deoxy-2-acetamido glucopyranoside, 4-nitrophenyl-α-D-mannopyranoside, dextran, gum carrageenan, gellan gum (Kelco), alginic acid, locust beam gum, or guar gum by crude extract.
[b] Average of three to five determinations.

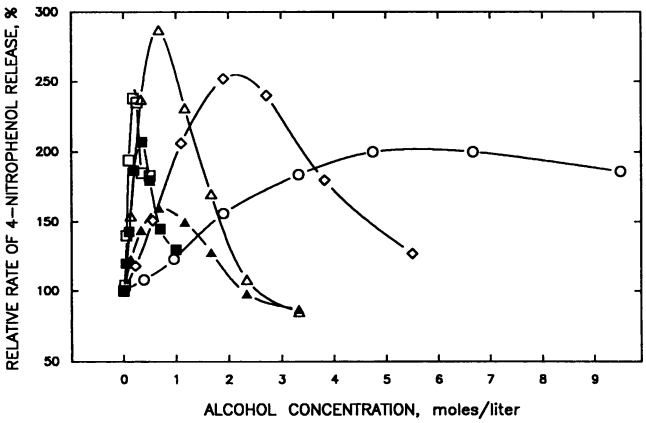

FIG. 2. Stimulation of β-glucosidase activity by alcohols. Purified enzyme (25 ng) was assayed in the standard mixture containing the indicated concentration of alcohol in a 0.5-ml-capacity, sealed tube submerged in water at 77°C (average of two determinations). Symbols: circles, methanol; diamonds, ethanol; triangles, propanol; squares, butanol; open symbols, primary alcohol; solid symbols, secondary alcohol.

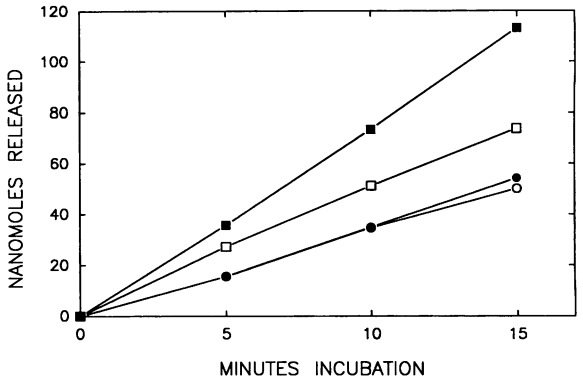

FIG. 3. Stoichiometry of salicin hydrolysis products. Aliquots (0.10 ml) contained 5 mg of salicin, 0.1 mg of gelatin, and 4.5 μg of purified enzyme per ml in nonchromogenic assay buffer as for Fig. 2 (see Materials and Methods). Symbols: circles, standard assay; squares, supplemented with 0.67 M 1-propanol; open symbols, reducing sugar; closed symbols, 2-(hydroxymethyl)phenol.

series became narrower and occurred at successively lower alcohol concentrations (Fig. 2).

(iii) Secondary alcohols exerted similar effects but yielded less total stimulation than the corresponding primary alcohol.

DISCUSSION

Extracts of all *Sulfolobus* isolates examined were able to hydrolyze β-glycosides at significant rates, and in *S. solfataricus* isolate P2 most of the β-glycosidase activities found in crude extracts could be attributed to a single enzyme. The present study provides conclusive evidence that the β-galactosidase and β-glucosidase activities derive from the same enzyme: (i) a variety of denaturing agents (high temperature, 3% [wt/vol] SDS, urea, low pH) destroyed both activities at the same rate, (ii) the ratio of the two activities remained constant during approximately 850-fold purification, (iii) the result of purification was an apparently homogeneous enzyme with very low electrophoretic mobility similar to that of both enzymatic activities, and (iv) substrates of the two activities competed for the same catalytic site. Hydrolysis of β-D-fucosides presumably occurs at this site also, considering the close structural analogy to β-D-galactosides. Comparison of the substrates hydrolyzed by crude extract and by purified β-glycosidase indicates at least one additional, distinct glycosidase in *S. solfataricus*, e.g., one hydrolyzing α-glucosides.

The β-glycosidase proved relatively ineffective against most polysaccharides tested, but its rather low specificity for both glycone and aglycone moieties of simple glycosides represents a possible advantage for certain applications. Removal of lactose from milk products has been proposed (3), but the higher catalytic efficiency toward β-glucosides demonstrated in this study suggests other possible uses, such as in the enzymatic conversion of cellulose to D-glucose (7). Comparison of literature values indicates that the *S. solfataricus* β-glycosidase has catalytic rates comparable to those of similar enzymes isolated from thermophilic eubacteria and is more thermostable. Examples of half-lives reported include β-galactosidase from *Bacillus stearothermophilus*, 7 min at 65°C (8); β-galactosidase from a *Thermus* species, 60 min at 88°C (24); and β-glucosidase from the thermophilic anaerobe "Tp8," 105 min at 85°C (20). These compare with >120 min at 87°C observed in this study for the *S. solfataricus* enzyme. The *S. solfataricus* β-glycosidase also proved more stable to polar solvent at high temperature than about 85% of total *S. solfataricus* protein, which was used to advantage in its purification (Table 2).

Apparent stimulation of β-glucoside hydrolysis by alcohols, observed here with the *S. solfataricus* enzyme, is a common, though not universal, feature of β-glucosidases (9, 20). The observed concentration dependence suggests binding of alcohol at a hydrophobic site which increases the rate of aglycone release at low concentrations but decreases it at high concentrations. Analysis of this effect in a mammalian enzyme has indicated that the increase is due to higher turnover rate in the presence of alcohol, whereas the decrease reflects competitive inhibition by the alcohol (9). One possible mechanism for the alcohol stimulation of aglycone release is preferential transfer of the glucosyl group to the alcohol rather than to water. The observed effect of 1-propanol on the stoichiometry of salicin hydrolysis products (Fig. 3) suggests that the *S. solfataricus* β-glycosidase can catalyze glucosyl transfer from the aryl aglycone to alcohol, although this does not account for all of the stimulation observed (Fig. 3). Another question requiring further study is the enzyme's function in vivo, which is almost certainly not the catabolism of lactose. In its geothermal habitat, *Sulfolobus* grows autotrophically via sulfur oxidation (2), and most laboratory strains do not effectively utilize β-linked disaccharides (10), yet all contain β-glycosidase activity. Also, growth of *S. solfataricus* P2 on D-cellobiose or lactose as sole carbon and energy source does not significantly induce the enzyme (data not shown). On the other hand, other data imply that β-D-glucosyl/β-D-galactosyl transferase should be required for the biosynthesis of membrane lipid in *S. solfataricus* (18).

Finally, it should be noted that *Sulfolobus* β-glycosidase can be expressed in *Escherichia coli*; this property has been used by two independent groups to clone and sequence the corresponding genes (5, 17). Although both published sequences have been attributed to *S. solfataricus*, comparison reveals a surprisingly low sequence homology. (At the time

of revision of the manuscript, a complete, computerized comparison of the two sequences was not feasible, as the sequence of Little et al. could not be located in the EMBL data collection under its published accession number, X15372.) For example, 34 mismatches occur within the first 120 nucleotides of the two genes and 37 occur in the last 120, yielding differences between 9 of the first 40 amino acid residues and 10 of the last 40 (5, 17). Thus, the sequence homology of these two functional genes seems significantly below the overall genomic homology (86 to 89%) between authentic *S. solfataricus* P1 and strain MT4 (12). It seems likely that the culture received by Little et al. under the designation DSM 1616 (17) contained the *S. acidocaldarius* of unknown origin also received from the same source under the same designation by Grote et al. (13) and by Grogan (10). Though somewhat confusing, this situation has the advantage of providing two distinct protein structures, which presumably share enzymatic function and thermostability, for detailed molecular comparison.

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