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GLUCOSYL TRANSFER BY BETA-THIOGLUCOSIDASE*

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Abstract—The β-thioglucosidase of black mustard, *Brassica nigra*, has been shown to carry out the transfer of a D-glucopyranosyl residue to an acceptor other than water. This appears to be the first reported example of transglycosylation catalyzed by a thioglucosidase system. The enzyme was partially purified by salt fractionation and the conditions determined for the hydrolysis of the donor substrate. *p*-Nitrophenyl-β-D-glucopyranoside was used as the glucosyl donor and glycerol as the acceptor molecule. The enzymatically synthesized glucoside was identified as 1-glyceryl-β-D-glucopyranoside. Thus it appears that the transglucosylation reaction has taken place with overall retention of the configuration of the donor molecule.

INTRODUCTION

THE ASSOCIATION of hydrolytic enzymes with the transfer of glycosyl residues to acceptors other than water was first demonstrated with an extract of the willow, *Salix purpurea*.[1] This activity, termed transglycosylation, is now known to exist in many systems and is closely related to the processes involved in the biosynthesis of glycosidic bonds.[2]

Almond emulsin β-glucosidase has long been known to catalyze transglucosylation reactions.[3] The similarity that exists between the emulsin enzyme and the thioglucosidase of mustard[4] suggested the possibility that the thioglucosidase may also carry out transglucosylation reactions.

This paper reports an initial observation of the transglucosylation reaction catalyzed by the β-thioglucosidase of black mustard, *Brassica nigra*, using *p*-nitrophenyl-β-D-glucopyranoside as a glucosyl donor and glycerol as an acceptor molecule.

RESULTS

Activity of the Enzyme

The enzyme fraction obtained by ammonium sulfate precipitation was taken up in acetate buffer and dialyzed against cold buffer solution for 24 hr. The final solution contained approximately three units of enzyme per ml of solution and represented a 150-fold increase in activity. One unit of enzyme was defined as the amount of enzyme necessary to liberate one micromole of *p*-nitrophenol in 30 min.

The purified enzyme was used to optimize conditions for the hydrolysis of *p*-nitrophenyl-β-D-glucopyranoside in acetate buffer. At 37°, the maximum activity was observed at pH

* Throughout the literature this name has been used synonomously with myrosinase and sinigrinase. The systematic name assigned to this enzyme is thioglucoside glucohydrolase (3.2.3.1).

[1] M. J. RABATE, *Bull. Soc. Chim. Biol.* **17**, 572 (1935).
[2] W. Z. HASSID and E. F. NEUFELD, In *The Enzymes* (edited by P. D. BOYER, H. LARDY and K. MYRBACK), Vol. 6, p. 277, Academic Press, New York (1959).
[3] J. E. COURTOIS and M. LECLERC, *Bull. Soc. Chim. Biol.* **38**, 365 (1956).
[4] R. D. GAINES and K. J. GOERING, *Arch. Biochem. Biophys.* **96**, 13 (1962).

7·5–8·5. The determinations of *p*-nitrophenol formed under these conditions indicate that the kinetics were zero order through approximately 70 per cent of hydrolysis and that the zero order velocity is directly proportional to the enzyme concentration.

Characterization of the Transglucosylation Product

The concentrated sirups from the chemical and enzymatic syntheses of the glycerol glucoside were chromatographed on paper using 2-methyl-2-butanol:*n*-propanol:water (4:1:1·5) as solvent. The chromatograms were developed with ammoniacal silver nitrate and gave identical R_f values for the glucosides.

The i.r. spectra of the concentrated sirups were compared and appeared to be identical. The spectra were run as thin films on a Perkin-Elmer Model 137 spectrophotometer.

The sirups were dried at 80° for 24 hr under vacuum[5] and compared by optical rotation and elemental analysis. For the enzymatically prepared glucoside, $[\alpha]_D^{20} -26·6°$ (c 5·0, water); reported: $[\alpha]_D^{20} -27·25°$ and $[\alpha]_D^{20} -26·2°$.[6, 5] The opitcal rotation for the chemically synthesized glucoside was $[\alpha]_D^{20} -27·2°$ (c 5·0, water); reported: $[\alpha]_D^{18} -27·7°$.[7] Found: C, 42·24; H, 7·0 (chemically synthesized) and C, 41·91; H, 7·12 (enzymatically synthesized). Calculated for $C_9H_{18}O_8$: C, 41·5; H, 7·1.

TABLE 1. PERIODATE OXIDATION OF GLYCERYL GLUCOSIDES

Glyceryl glucopyranoside	Oxidant consumed mole/mole	Formic acid formed mole/mole	Formaldehyde formed mole/mole
Enzymatic[a]	2·95	0·98	0·97
Chemical[a]	2·96	0·97	0·98
1-Glyceryl-[b]	3·00	1·00	1·00
2-Glyceryl-[b]	2·00	1·00	0

[a] Experimental values; [b] Calculated values

In order to establish whether a primary or secondary hydroxyl of glycerol was involved in the glucosidic bond, the sirup samples were subjected to periodate oxidation. These results are summarized in Table 1. The values obtained agree with those calculated for 1-glyceryl-β-D-glucopyranoside, although they do not rule out slight contamination by the 2-isomer.

It should be noted that the formation of 1-glyceryl-β-D-glucopyranoside has created a new asymmetric center at the 2-position of glycerol. Bourquelot *et al.*[6] and Jermyn[5] suggest that the products are mixtures of the two enantiomers in unknown proportions. Thus the measured optical rotations are not criteria for purity, but serve only for comparison with reported values. No attempt was made to resolve the isomeric mixtures.

DISCUSSION

The results indicate that during the hydrolysis of *p*-nitrophenyl-β-D-glucopyranoside by mustard β-thioglucosidase, transglucosylation to acceptor molecules other than water may

[5] M. A. JERMYN, *Australian J. Biol. Sci.* **11**, 114 (1958).
[6] E. BOURQUELOT, M. BRIDEL and A. AUBRY, *Compt. Rend.* **160**, 823 (1915).
[7] P. KARRER and O. HURWITZ, *Helv. Chim. Acta* **5**, 864 (1922).

take place. As mentioned previously, transglucosylation reactions catalyzed by hydrolytic enzymes are not unusual, but this appears to be the first instance in which such a reaction has been shown to take place in a thioglucosidase system.

It is difficult to distinguish between a β-glucosidase and a β-thioglucosidase on the basis of the glycosidic linkage hydrolyzed. Enzymes of both classifications have frequently been shown to hydrolyze substrates containing either sulfur or oxygen in the glycosidic bond.[4, 8–10] In this respect it would appear likely that the β-thioglucosidase may also transfer a glucosyl residue from a thioglucopyranosyl donor to an oxygen acceptor. The possibility of transglucosylation to nitrogen or sulfur acceptors has not been explored for the thioglucosidase system, nor have the ranges of donor or acceptor molecules been established.

The data indicate that the enzymatically synthesized glucoside is 1-glyceryl-β-D-glucopyranoside and that the transglucosylation reaction has retained the beta configuration of the donor molecule.

The retention of configuration in transfer reactions has been explained as a double displacement mechanism involving an inversion at each transfer.[11] For this reaction such a mechanism would involve the formation of an α-D-glucopyranosyl-enzyme and subsequent displacement by glycerol to give 1-glyceryl-β-D-glucopyranoside.

There are, however, other proposed mechanisms consistent with retention of configuration[12, 13] and until further evidence is obtained, these mechanisms cannot be ruled out.

EXPERIMENTAL

Methods and Materials

The β-thioglucosidase was prepared from defatted mustard seed by ammonium sulfate fractionation of the water soluble extract. The hydrolysis of sinigrin, the natural substrate of the β-thioglucosidase from *Brassica nigra*, was used to follow the isolation and purification of the enzyme. Qualitative protein analysis was measured by absorption at 280 nm and quantitative protein analysis by the method of Lowry[14] using a serum albumin standard.

Sinigrin, glucotropaeolin and *p*-nitrophenyl-β-D-glucopyranoside were purchased from Calbiochem, Los Angeles, California. The latter substrate was recrystallized to a constant m.p., 172° (uncorrected), and had no appreciable absorption at 400 nm.

For analysis of activity 1·5 mg of substrate in 10·0 ml of buffer was incubated with 0·1 ml of enzyme solution. Acetate buffer, pH 7·0, was used for both enzyme and substrate solutions. The reaction vessels were sealed and incubated at 37° for 30 min. The reactions were stopped by heating at 100° for 2 min. (Heating for a longer period caused considerable degradation of some substrates and resulted in very high blank values.) Activity was then measured by the amount of *p*-nitrophenol liberated as determined by absorption at 400 nm[15] or by the increase in reducing sugar measured by the reduction of 3,5-dinitrosalicylic acid.[16]

[8] T. Reese, R. C. Clapp and M. Mandels, *Arch. Biochem. Biophys.* **75**, 228 (1958).
[9] T. Goodman, J. R. Fouts, E. Bresnick, R. Menegas and G. H. Hitchings, *Science* **130**, 450 (1959).
[10] G. Wagner and R. Metzner, *Naturwissenschaften* **52**, 61 (1965).
[11] D. E. Koshland, Jr., In *The Enzymes* (edited by P. D. Boyer, H. Lardy and K. Myrback), Vol. 6, p. 277, Academic Press, New York (1959).
[12] M. A. Jermyn, *Science*, **125**, 12 (1957).
[13] R. C. Mayer and J. Larner, *J. Am. Chem. Soc.* **81**, 188 (1959).
[14] O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
[15] J. D. Duerksen and H. Halvorson, *J. Biol. Chem.* **233**, 1113 (1958).
[16] P. Bernfeld, In *Methods in Enzymology* (edited by S. P. Colowick and N. O. Kaplan), Vol. I, p. 149, Academic Press, New York (1955).

Glyceryl-β-D-glucopyranoside has been previously prepared;[7] however, a more convenient method was to react equivalent amounts of tetra-*O*-acetyl-α-D-glucopyranosyl bromide with the sodium salt of glycerol. Stoiciometric quantities of clean, finely-cut Na and glycerol were combined in 15–20 ml acetone. The reaction was run under N_2 and stirred until all the Na had dissolved. An equivalent amount of the glucosyl halide dissolved in acetone was added to the Na glycerate solution. Stirring was continued for about 24 hr. NaBr was removed by filtration, and the filtrate concentrated to a viscous sirup. The glucosidic product was deacylated using sodium methoxide in methanol and purified by adsorption on charcoal as described below.

Purification of the Enzyme

The clear water-soluble extract of the pulverized, defatted seeds was saturated with enzyme-grade ammonium sulfate and allowed to stand for several days at 4°. The precipitated protein was collected by centrifugation and the supernatent discarded. The protein was taken up in a minimum of cold, distilled water and dialyzed against cold, running water for 12 hr. Appropriate amounts of solid $(NH_4)_2SO_4$ were added to the dialyzed solution to increase the saturation of the solution by 10% increments. That fraction collected from 50–60% saturation appeared to contain most of the activity and was retained. The thioglucosidase system, purified by this method, showed good activity toward sinigrin, glucotropaeolin and *p*-nitrophenyl-β-D-glucopyranoside and thus further purification was not attempted for this study.

Transglucosylation

Fifty units of the purified β-thioglucosidase were added to a solution containing 500 mg of *p*-nitrophenyl-β-D-glucopyranoside and 3·5 ml glycerol in acetate buffer. The solution was diluted to 50 ml with buffer and allowed to incubate at 37° for 24 hr. Reagent blanks and controls were run simultaneously. All reactions were run under aseptic conditions in order to prevent external contamination.

Each glycosidic solution was stirred with 10 g of activated charcoal. The charcoal was washed repeatedly with water and finally with 50% ethanol to elute the glyceryl-D-glucoside. The glucoside solution was concentrated under vacuum to yield a sirupy material which could not be induced to crystallize.