BIOSYNTHESIS OF 6-β-GALACTINOL BY β-GALACTOSIDASE FROM RAT MAMMARY GLAND AND *E. coli*[1]

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SUMMARY: The synthesis of 6-β-galactinol by rat mammary gland extracts *in vitro*, was shown to be catalyzed by β-galactosidase (EC 3.2.1.23) by transferring the galactosyl group of lactose (K_m = 37 mM) to *myo*-inositol (K_m = 380 mM) with a pH optimum of 3.6. Highly purified commercial β-galactosidase from *E. coli* catalyzed a similar reaction yielding 6-β-galactinol from lactose (K_m = 20 mM) and *myo*-inositol (K_m = 770 mM) at a pH optimum of 7.2. The identity of 6-β-galactinol synthesized by the action of β-galactosidase from either source was verified by gas chromatography and mass spectrometry of the fully trimethylsilylated disaccharide.

INTRODUCTION.

A disaccharide, recently isolated from rat mammary gland and milk, was identified as 6-O-β-D-galactopyranosyl *myo*-inositol (6-β-galactinol) (1, 2). An enzyme has now been partially purified from rat mammary tissue that catalyzes the transfer of galactose from lactose to *myo*-inositol to produce a compound with the properties of 6-β-galactinol. During the purification of the enzyme, a constant ratio between 6-β-galactinol synthesis (transferase) and β-galactoside hydrolase activity was observed. In this communication, we present selected physical and kinetic characteristics of the transferase activity of rat mammary β-galactosidase (EC 3.2.1.23). These properties are compared with those of highly purified *E. coli* β-galactosidase obtained from a commercial source.

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MATERIALS AND METHODS.

Fresh rat mammary gland was obtained from lactating (18th day) Sprague Dawley rats (Spartan Research, Haslett, Mi.). The mammary tissue was removed after decapitation and stored at -20°C. Mammary tissue (45.6 g) was homogenized (Tekmar Model SDT) in 90 ml of 50 mM Tris-HCl buffer, pH 7.5, containing 0.25 M sucrose at 0-4°C. For subsequent purifications, the procedure of Meisler for liver β-galactosidase was followed through the DEAE cellulose step (3). The fractions obtained during the purification were assayed for both β-galactoside hydrolase and transferase activity. *E. coli* β-galactosidase (30 units/mg) was a product of Boehringer-Mannheim, New York N.Y.

Hydrolase Activity. The hydrolase activity of β-galactosidase was determined as previously described (4) using *p*-nitrophenyl-β-D-galactopyranoside as substrate. The reaction mixture, consisting of 50 mM sodium phosphate-citrate buffer, pH 5.0, 3 mM *p*-nitrophenyl-β-D-galactopyranoside and enzyme in a final volume of 500 µl, was incubated at 37°C for 1 hr. The *p*-nitrophenolate ion liberated was determined at 410 nm after adjusting the pH to 9.3 with Na borate-NaOH (0.108 M $Na_2B_4O_7 \cdot H_2O$ - 0.133 N NaOH), $\varepsilon_{410\ nm}$ = 15.5 $cm^2/\mu mole$.

Lactose: *myo*-Inositol Galactosyl Transferase Activity. For the mammary enzyme, the reaction mixture contained 50 mM Na acetate buffer, pH 3.6, 175-200 mM lactose, 250-375 mM *myo*-inositol, and enzyme in a final volume of 200 µl. The reaction was incubated at 37°C for 4.0 hr and a 50 µl aliquot of the reaction mixture was terminated by the addition of 0.3 ml of 0.3 N $Ba(OH)_2$ and 0.3 ml of 5% $ZnSO_4$ ($7H_2O$). A known amount of standard trehalose (Sigma Chemical Co., St. Louis, Mo.) was added prior to centrifugation at 1,600 x g for 5 min. An aliquot of the sample was dried at 40°C in a rotary evaporator, trimethylsilylated with standard TMSi reagent (5), and quantified by gas-liquid chromatography. The samples were separated at 195°C on a 1.8 m x 3 mm glass column packed with 3% XE-60 on Gas-Chrom Q, 100-200 mesh

(Applied Science Laboratories, Inc., State College, Pa.). The amount of product formed was linear with respect to enzyme concentration and time up to 9 hr. No product was formed when either *myo*-inositol, lactose, or enzyme was omitted.

E. coli β-Galactosidase and 6-β-Galactinol Synthesis. Transferase activity of the *E. coli* enzyme had a pH optimum of 7.2. The routine assay consisted of 50 mM Tris-maleate buffer, pH 7.2, 167.5 mM lactose, 560 mM *myo*-inositol, and enzyme (6.0 μg) in a final volume of 0.2 ml and was incubated at 37°C for 15 min. The formation of 6-β-galactinol was linear with respect to enzyme concentration and reaction time up to 1 hr. Protein was determined by the method of Lowry *et al.* (6) using bovine serum albumin as the standard.

Identification of Product. Combined gas-liquid chromatography-mass spectrometry of the trimethylsilylated product of the enzyme-catalyzed reaction was recorded at an electron energy of 70 eV with a LKB 9000 mass spectrometer. The relative abundance of fragments was displayed as bar graphs by means of an on-line data acquisition and processing program (7). The source temperature was 290°, accelerating voltage 3.5 KV, and the ionizing current 60 μA. Sample introduction was via the GC inlet using a 3 mm x 1.8 m glass coil packed with 3% XE-60 on Gas Chrom Q, 80-100 mesh. The column temperature was 190°C.

RESULTS AND DISCUSSION.

The purification of β-galactosidase resulted in a 22-fold increase in the hydrolase activity and a 27-fold increase in the transferase activity (Table I). Throughout the purification, the ratio of hydrolase/transferase activity varied by approximately 10%. The product of the transferase reaction was shown to be 6-β-galactinol by comparison of the mass spectra of the trimethylsilylated compounds resulting from both mammary and *E. coli* β-galactosidase activity with that of authentic 6-β-galactinol previously isolated in this laboratory (1, 2). Whole rat milk contained both hydrolase

TABLE 1. Comparison of Hydrolase and Transferase Activities of β-Galactosidase Partially Purified from Rat Mammary Gland[a]

Fraction	Total Vol. ml	Protein Conc. mg/ml	Total Protein mg	Total Units *p*-NP[b] μmole/min	Total Units 6-β-G[b] μmole/min	Sp. Activity *p*-NP nmole/min/mg	Sp. Activity 6-β-G nmole/min/mg	Sp. A. *p*-NP / Sp. A. 6-β-G
Crude Homogenate	107	36.2	3,870	7.554	3.329	1.95	0.86	2.27
1st $(NH_4)_2SO_4$ ppt.	36.3	50.8	1,840	4.520	2.369	2.46	1.29	1.91
Solvent ppt.	37.3	5.4	201	0.740	0.414	3.68	2.06	1.79
2nd $(NH_4)_2SO_4$ ppt.	2.8	51.7	145	0.715	0.352	4.93	2.43	2.03
DEAE-Cellulose	0.9	5.7	5	0.213	0.116	42.60	23.20	1.84

[a]The assay conditions are described under the Materials and Methods section.

[b]*p*-NP and 6-β-G are abbreviations for hydrolase activity using *p*-nitrophenyl β-D-galactopyranoside as substrate and 6-β-galactinol synthetase activity of β-galactosidase, respectively.

(16.2 nmoles *p*-nitrophenol released/min/ml) and transferase activity (15.4 nmoles 6-β-galactinol produced/min/ml) of β-galactosidase.

The pH optimum of the reaction catalyzed by the mammary enzyme was 3.6, whereas that from *E. coli* was 7.2, though in either case, the pH optima for hydrolase and transferase activities were similar. The apparent K_m for *myo*-inositol for the transferase activity from mammary gland and *E. coli* were 380 and 770 mM, respectively, while the corresponding values for lactose were 37 and 20 mM. Though these constants are high, the lactose concentration in rat milk determined by GLC (5) is 70 mM. The availability of *myo*-inositol is, therefore, rate limiting for the synthesis of 6-β-galactinol, *in vivo*, and correlates well with the developmental pattern of *myo*-inositol secretion during lactation (2).

Initial attempts to find enzymatic activity in rat mammary gland capable of synthesizing 6-β-galactinol were patterned after the galactosyl

transferase activity for 1-α-galactinol biosynthesis observed in peas by Frydman and Neufeld (8). The plant enzyme requires UDP-galactose as galactosyl donor. We were unsuccessful in demonstrating analogous activity in rat mammary homogenates. When it became evident that 6-β-galactinol occurred only in mammary tissue or in milk (2), lactose was considered a likely candidate for the immediate galactosyl donor. As early as 1952, Aronson (9) had described the transgalactosylation from lactose to various carbohydrates as a function of the β-galactosidase activity from *Saccharomyces fragilis* and *E. coli*. Subsequently, this activity of β-galactosidase was investigated by numerous laboratories (10-15). Moreover, Gorin *et al.* (16), showed that when *myo*-inositol was tested as an acceptor of galactosyl transfer from lactose under the influence of an enzyme extract from *Sporobolomyces singularis*, the major transferase product (74%) was 5-O-β-D-galactopyranosyl *myo*-inositol. In the study reported herein, partially purified β-galactosidase from rat mammary gland catalyzed the formation of 6-O-β-D-galactopyranosyl-*myo*-inositol and a second major isomer whose retention time on the XE-60 column is significantly shorter than that of 6-β-galactinol, but whose mass spectrum of the fully trimethylsilylated derivative is identical with that of 6-β-galactinol. The unknown was not observed in mammary tissue nor in rat milk and was not observed following incubation of the *E. coli* β-galactosidase at pH 7.2 under standard conditions. The competing activities of this hydrolase acting on lactose with either H_2O, *myo*-inositol, or X (where X may be lactose, glucose, or galactose) as acceptors of galactose may be outlined as follows:

1) lactose + Enz $\rightleftarrows$ galactosyl-Enz + glucose

2) galactosyl-Enz + $H_2O \rightarrow$ galactose + Enz

3) galactosyl-Enz + *myo*-inositol $\rightleftarrows$ 6-β-galactinol + Enz

4) galactosyl-Enz + X $\rightleftarrows$ galactosyl-X + Enz

In summary, we conclude that β-galactosidase isolated from rat mammary

gland or obtained commercially from *E. coli* possesses transferase activity capable of yielding 6-β-galactinol. Thus, 6-β-galactinol occurs in those biological systems that include lactose, *myo*-inositol, and β-galactosidase. Further purification of the mammary enzyme and comparison of its properties with those of β-galactosidase from other mammalian tissues and species are under investigation.

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REFERENCES

1. Naccarato, W.F., and Wells, W.W. (1974) *Biochem. Biophys. Res. Commun.* 57, 1026-1031.
2. Naccarato, W.F., Ray, R.E., and Wells, W.W. (1975) *J. Biol. Chem.* 250, 1872-1876.
3. Meisler, M. (1972) *Methods Enzymol.* 28, 820-824.
4. Blosser, J.C., and Wells, W.W. (1972) *J. Neurochem.* 19, 1539-1547.
5. Sweeley, C.C., Wells, W.W., and Bentley, R. (1966) *Methods Enzymol.* 8, 95-108.
6. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) *J. Biol. Chem.* 193, 265-275.
7. Holland, J.F., Sweeley, C.C., Thrush, R.E., Teets, R.E., and Bieber, M.A. (1973) *Anal. Chem.* 45, 308-314.
8. Frydman, R.B., and Neufeld, E.F. (1963) *Biochem. Biophys. Res. Commun.*, 12, 121-125.
9. Aronson, M. (1952) *Arch. Biochem. Biophys.* 39, 370-378.
10. Wallenfels, K., Bernt, E., and Limberg, G. (1954) *Ann.* 584, 63-85.
11. Kuhn, R. (1958) *Bull. Soc. Chim. Biol.* 40, 297-314.
12. Alessandrini, A., Schmidt, E., Zilliken, F., and Gyorgi, P. (1955) *J. Biol. Chem.* 220, 71-78.
13. Wallenfels, K., Zarnitz, M.L., Laule, G., Bender, H., and Keser, M. (1959) *Biochem. Z.* 331, 459-485.
14. Ballio, A., and Russi, S. (1960) *J. Chromatog.* 4, 117-129.
15. Gorin, P.A.J., Spencer, J.F.T., and Phaff, H.J. (1964) *Can. J. Chem.* 42, 2307-2317.
16. Gorin, P.A.J., Horitsu, K., and Spencer, J.F.T. (1965) *Can. J. Chem.* 43, 2259-2264.