

Improved Purification of Rat Intestinal Lactase

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Abstract. A rapid and improved method to obtain purified lactase from rat intestine is described. The purification procedure involved only two chromatographic steps. The degree of purification was far above (500 fold) the values reached with classical methods. Rabbit antisera raised to the purified lactase were characterized using conventional immunological techniques. The specificity of the lactase antibodies was confirmed by the lack of interference on maltase, aminopeptidase and alkaline phosphatase activities measured after papain extraction of the membrane proteins.

Key words: Rat — Intestine — Lactase — Antibodies — Lactase purification

Introduction

Lactase (EC 3.2.1.23) is associated with phlorizin hydrolase (EC 3.2.1.62) in the form of a dimeric complex specifically anchored in the intestinal microvillus membrane. Lactase represents the main disaccharidase activity in the brush border membrane of the suckling rat. The enzyme exhibits maximal activity shortly after birth and low activity after weaning (Doell and Kretchmer 1962; Henning and Kretchmer 1973). Lactase has attracted much attention since low levels of enzyme activity have been found under normal conditions in the intestinal mucosa of the major part of the adult population in the world. Intestinal hypolactasia causes clinical symptoms following the ingestion of milk and is also a common feature associated with intestinal diseases like coeliac disease or gastroenteritis (Auricchio et al. 1963; Dahlqvist et al. 1963; Freiburghaus et al. 1976; Sahi 1978; Simoons 1978). We describe here a convenient method for the purification of lactase from suckling rat intestine by two chromatographic steps following papain extraction of brush border membrane proteins. By this procedure lactase of a high purity was obtained and used for the preparation of specific antisera. The specificity of the antisera was measured by conventional immunological techniques and by the ability of the membrane-bound antibodies to interfere with the papain extraction of the brush border enzymes.

Materials and Methods

Enzyme preparation: The small intestine extending from the ligament of Treitz to mid ileum was excised from 15 day old Wistar rats. The segments were opened and washed with 0.9% NaCl and homogenized in 50 mmol/l mannitol, 2 mmol/l Tris, pH 7.1. The brush border membranes were isolated from mucosal homogenate as described previously (Schmitz et al. 1973; Raul et al. 1978). The pellet containing the purified brush border membranes was resuspended in 10 mmol/l potassium phosphate buffer pH 6.0 (Buffer A). The membranes were then incubated with insoluble papain (Sigma Chemical Co.) at a concentration of 10 µg papain per mg protein for 35 minutes at 37 °C. After centrifugation of the samples at 20,000xg for 30 minutes, the resulting supernatant containing the solubilized proteins was fractionated on a 1.5 × 100 cm column of agarose (Bio-Gel, A-15 m; Bio-Rad) at a flow rate of 9 ml/h. The elution medium was buffer A. A major peak of lactase was eluted at 112 ml elution volume. The fractions of the lactase peak were fractionated further on a 1 × 9 cm column of hydroxyapatite (HAUltrogel; IBF). The column was washed with buffer A. Lactase was retained and eluted at a concentration of 150 mmol/l phosphate buffer. The lactase fractions were dialyzed in buffer A and concentrated.

Enzyme assays: Lactase activity was measured in the presence of *p*-chloromercuribenzoate (Koldovsky et al. 1969), maltase (EC 3.2.1.20) was assayed according to Messer and Dahlqvist (1966). Aminopeptidase (EC 3.4.11.2) was determined with L-alanine-*p*-nitroanilide as substrate (Maroux et al. 1973) and alkaline phosphatase (EC 3.1.3.1.) was measured with *p*-nitrophenyl-phosphate as substrate (Garen and Levinthal 1960). One unit of enzyme activity equals 1 µmol of product formed per minute at 37 °C.

Immunological procedures: Rabbits were immunised by subcutaneous injection of 50 µg purified lactase mixed with 0.5 ml complete Freund's adjuvant. The injection was repeated once 3 weeks later. Bleedings were made three weeks after the last injection and the sera were tested by the ELISA technique for antibody titers. All the immunological reactions were performed in 10 mmol/l sodium phosphate at pH 7.4 (Clark and Adams 1977). Competition assays were carried out by varying the amounts of competitor (lactase or maltase) to be incubated with various dilutions of rabbit serum under conditions of excess antigen on the plate. The antigen-antibody complex was revealed by incubation with sheep anti-rabbit antibodies linked with peroxidase. Peroxidase activity was measured in the presence of 0.01% H₂O₂ and 1 mg/ml ABTS ((2,2'-azino)-di-[3-ethylbenzthiazolin-sulfonate]); Boehringer Mannheim) as substrates (Clark and Adams 1977).

Results and Discussion

Enzyme purification: The clear solubilized protein solution obtained after papain extraction of the brush border membrane was directly applied on a column of agarose. Agarose was chosen since its matrix is made of polysaccharides with β_{1-4} glycosidic bounds. Lactase which is the only β -glycosidase of the brush border membrane should normally be retarded by interactions with the matrix and separated from the α -glycosidases despite similar molecular weights of all these enzymes. Indeed, the solution profile showed that maltase was eluted before lactase (Fig. 1a). Most of the maltase was well separated from lactase, however some overlapping of maltase was always present in the peak of lactase. Lactase was then selectively adsorbed on a hydroxyapatite column and subsequently eluted at

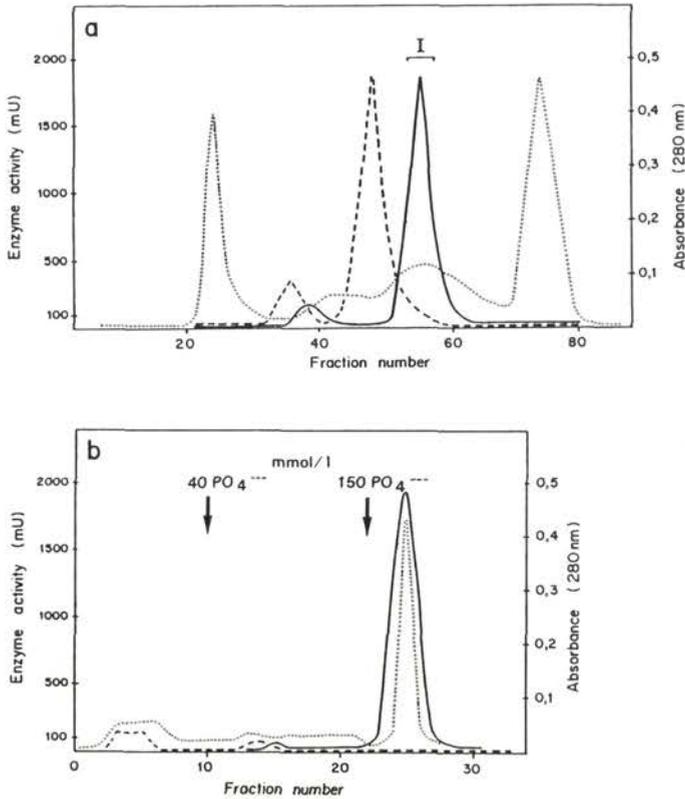


Fig. 1. (a) Fractionation of brush border membrane proteins on agarose column (Bio-Gel, A-15m) (b) Fractionation of peak I corresponding to lactase activity on hydroxyapatite Ultrogel column. The absorbance was measured at 280 nm (.....). Activities of lactase (—) and maltase (---) were determined in the eluted fractions.

a concentration of 150 mmol/l potassium phosphate buffer (Fig. 1b). A summary of yields and purification steps for intestinal lactase is given in Table 1. It is noteworthy that a 500 fold purification was obtained for lactase with this procedure. The degree of purification is far above those obtained by other methods (Schlegel-Haueter et al. 1972; Tsuboi et al. 1979; Cousineau and Green 1980; Goda et al. 1984). The ratio between lactase and the other proteins is higher in the purified brush border membrane when compared to that found in whole homogenate. Thus, our improvement may arise from the use of purified brush border membranes for papain extraction instead of mucosal homogenates. The proteins obtained at the various purification steps were analysed by SDS polyacrylamide slab gel electrophoresis (Fig. 2). Lactase which is one of the main proteins

Table 1. Purification of lactase from the intestine of 15 day old suckling rats.

Fraction	Volume (ml)	Protein (mg)	Lactase		
			Total Units	Specific Activities	Recovery (%)
Homogenate	48.00	1253	31.55	0.02	100.00
Brush border membrane	13.50	58.86	11.71	0.19	37.00
Papainized extract	5.90	12.45	4.43	0.35	14.00
Bio-Gel, Conc	1.44	2.02	4.36	2.16	13.80
Hydroxyapatite Conc	0.85	0.42	4.26	10.14	13.5

Each enzyme preparation comprised 22 to 24 infant rats. The starting material corresponded to 10–15 g of freshly excised small intestine.

Table 2. Lack of interference by bound antilactase on maltase, aminopeptidase and alkaline phosphatase extraction with papain from the brush border membranes.

Membrane treatment	Enzyme specific activities (mU/mg prot)			
	Lactase	Maltase	Amino-peptidase	Alkaline phosphatase
Membrane alone	119	148	52	856
Membrane + bound antilactase	0.70	146	48	847

Aliquots of membrane (50 µg membrane proteins) were placed in two sets of tubes and incubated either with excess antilactase (5 µl antiserum) or with control serum (5 µl serum) for 18 h at 4 °C in 500 µl medium containing 0.1% serum albumin, 10 mmol/l sodium phosphate, 5% Tween 20, pH 6.0. Proteins were extracted from the brush border membranes by papain digestion. The resulting extracts were assayed for lactase, maltase, aminopeptidase and alkaline phosphatase activities.

in the brush border membrane of the suckling rat (Fig. 2B), showed a high degree of purity after isolation on hydroxyapatite (Fig. 2D).

Immunological studies: Purified lactase yielded from hydroxyapatite column was injected into rabbits for immunization. To characterize the reactivity of the rabbit antiserum with lactase a number of standard immunological techniques were initially employed. Using rocket immunoelectrophoresis or Ouchterlony assays, a distinct immunoprecipitin line was visible with partially or completely purified preparations of lactase. No precipitin lines were observed with a purified prepara-

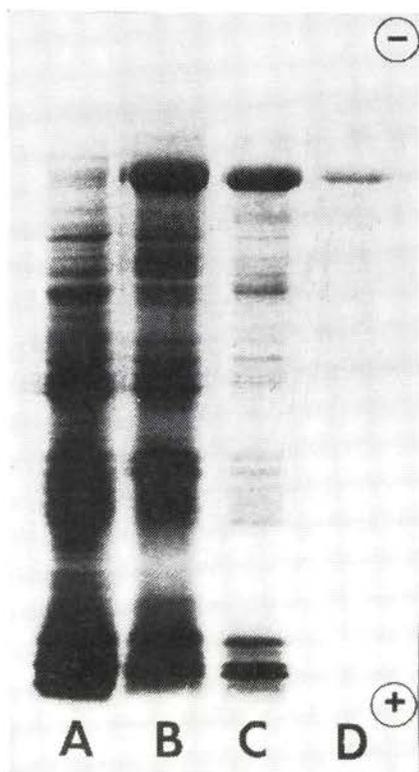


Fig. 2. Protein gel electrophoresis on 5–15% SDS polyacrylamide slab gel (Rochette-Egly et al. 1984). Protein samples were denatured by boiling for 5 min in 12 mmol/l Tris HCl buffer (pH 8.5), 1% SDS, 0.1% dithioerythritol (DTE). The gels were stained with Coomassie Blue. A: mucosal homogenate (150 μ g protein) B: brush border membrane proteins (150 μ g protein) C: papainized extract of the brush border membrane (75 μ g protein) D: purified lactase (15 μ g protein).

tion of maltase (unpublished observations). Figure 3 shows a typical standard inhibition curve obtained with various concentrations of lactase ranging from 1 to 1,000 ng/ml. The useful portion of the S-shaped inhibition curve extended from about 25 to 250 ng lactase/ml. This kind of inhibition curve may be used to accurately measure relative lactase concentrations in biological samples using purified rat intestinal lactase as a standard. No inhibition of antibody binding was observed when purified maltase was used instead of lactase. The extraction properties by papain of various enzymes from the brush border membrane containing bound antilactase are illustrated in Table 2. Preloading the brush border membrane with antilactase inactivated specifically lactase but had no effects on the extraction of maltase, aminopeptidase and alkaline phosphatase from the mem-

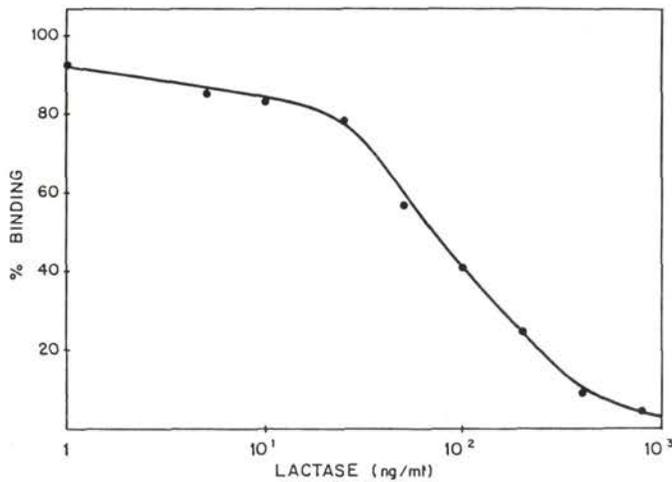


Fig. 3. Competition between bound and free lactase against antilactase. Samples of competitor proteins ranging from 1 ng to 1 μ g/ml were incubated with diluted rabbit antiserum (1/1000e) for 120 min at 20 °C. Samples were further processed as outlined under "Materials and Methods".

brane. These results were in agreement with those obtained by Tsuboi et al. (1979) with brush border lactase and maltase and were extended in the present study to other enzymes not related to disaccharide hydrolysis, confirming the high specificity of lactase antisera.

Acknowledgements. Mrs. F. Gossé and M. T. Vanier are gratefully acknowledged for their excellent technical assistance.

References

- Auricchio S., Rubino A., Landolt M., Semenza G., Prader A. (1963): Isolated intestinal lactase deficiency in the adult. *Lancet* **2**, 324—326
- Clark M. F., Adams A. N. (1977): Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. *J. Gen. Virol.* **34**, 475—483
- Cousineau J., Green J. R. (1980): Isolation and characterization of the proximal and distal forms of lactasephlorizin hydrolase from the small intestine of the suckling rat. *Biochim. Biophys. Acta* **615**, 147—157
- Dahlqvist A., Hammond J. B., Crane R. K., Dunphy J. V., Littman A. (1963): Intestinal lactase deficiency and lactose intolerance in adults. *Gastroenterology* **45**, 488—491
- Doell R. G., Kretchmer N. (1962): Studies of the small intestine during development. I. Distribution and activity of B-galactosidase. *Biochim. Biophys. Acta* **62**, 353—362
- Freiburghaus A. U., Schmitz J., Schindler M., Rotthauwe H. M., Kuitunen P., Launiala K., Hadorn B. (1976): Protein patterns of brush-border fragments in congenital lactose malabsorption and in specific hypolactasia of the adult. *N. Engl. J. Med.* **294**, 1030—1032

- Garen A., Levinthal C. (1960): A fine structure genetic and chemical study of the enzyme alkaline phosphatase of *E. Coli*. I. Purification and characterization of alkaline phosphatase. *Biochim. Biophys. Acta* **38**, 470—483
- Goda T., Bustamante S., Thornburg W., Koldovsky O. (1984): Dietary-induced increase in lactase activity and in immunoreactive lactase in adult rat jejunum. *Biochem. J.* **221**, 261—263
- Henning S. J., Kretchmer N. (1973): Development of intestinal function in mammals. *Enzyme* **15**, 3—23
- Koldovsky O., Asp N. G., Dahlqvist A. (1969): A method for the separate assay of "neutral" and "acid" B-galactosidase in homogenates of rat small intestinal mucosa. *Anal. Biochem.* **27**, 409—418
- Maroux S., Louvard D., Baratti J. (1973): The aminopeptidase from dog intestinal brush-border. *Biochim. Biophys. Acta* **321**, 282—295
- Messer M., Dahlqvist A. (1966): A one step ultramicromethod for the assay of intestinal disaccharidases. *Anal. Biochem.* **14**, 376—392
- Raul F., Simon P. M., Kedinger M., Grenier J. F., Haffen K. (1978): Separation and characterization of intestinal brush-border enzymes in adult rats and in suckling rats under normal conditions and after hydrocortisone injections. *Enzyme* **23**, 89—97
- Rochette-Egly C., Stussi-Garaud C. (1984): Selective detection of calmodulin in polyacrylamide gels by double staining with Coomassie Blue and silver. *Electrophoresis* **5**, 285—288
- Sahi T. (1978): Dietary lactose and the aetiology of human small intestinal hypolactasia. *Gut* **19**, 1074—1086
- Schlegel-Haueter S., Hore P., Kerry K. R., Semenza G. (1972): The preparation of lactase and glucoamylase of rat small intestine. *Biochim. Biophys. Acta* **258**, 506—519
- Schmitz J., Preiser H., Maestracci D., Ghosh B. K., Cerda J. J. (1973): Purification of human intestinal brush-border membrane. *Biochim. Biophys. Acta* **92**, 305—311
- Simoons F. J. (1978): New light on ethnic differences in the adult lactose intolerance. *Amer. J. Dig. Dis.* **23**, 963—980
- Tsui K. T., Schwartz S. M., Burrill P. H., Kwong L. K., Sunshine P. (1979): Sugar hydrolases of the infant rat intestine and their arrangement on the brush-border membrane. *Biochim. Biophys. Acta* **554**, 234—235

Received April 4, 1985 / Accepted June 24, 1985