PURIFICATION OF CALF INTESTINAL LACTASE*

by

ROGER G. YOUNG** AND F. J. REITHEL

Department of Chemistry, University of Oregon, Eugene, Oregon (U.S.A.)

Recent attempts to develop a specific and sensitive method for lactose[1] have indicated the possibility of using the enzyme lactase to good advantage. The utility of this method depends, however, on the availability of a purified preparation whose enzymic impurities, which might interfere, are known. A survey of the literature revealed no studies eventuating in the isolation of a highly active lactase preparation, although the occurrence of such activity has been noted to be widely distributed in biological materials. It has long been known that the intestinal mucosa of young animals contains considerable lactase activity[2,3] and this was chosen as a source material since it was readily available in quantity. In the following the purification procedures developed are described. Specificity studies will be reported at a later date.

METHODS

Tissues

Intact small intestines of calves were obtained within a few hours of killing. The outer fat was removed and the intestinal contents flushed out with a stream of water. A length of about 40 feet measured from the pylorus (containing virtually all the lactase) was stored in tin plate cans at — 10° C until ready for use.

Fetal calf intestines were stored similarly. The whole small intestines were used. A dried partially purified preparation of bovine intestinal phosphatase (Armour), containing lactase as an impurity, was used for some purification experiments. No precautions were taken in storing this prior to use.

Protein analysis

The protein content of crude extracts containing lactase was measured by the procedure of GORNALL *et al.*[4] Standard curves were prepared by analysis of lyophilized crude preparations. Purified preparations were dialysed, lyophilized and weighed directly, or analysed by the method of BUCHER[5] adapted to a photoelectric nephelometer.

Enzyme assay

In estimating the activity of lactase, aliquots of the enzyme solution were incubated at 37° C with lactose at a concentration of 0.03 *M*, and phthalate buffer pH 6.0 at a concentration of 0.12 *M*. Sufficient enzyme was used to effect 10–20% hydrolysis in one hour. At the end of the incubation, enzyme action was halted by heating the samples to 100° C for three minutes.

The extent of hydrolysis was estimated by determining the glucose formed. Glucose was determined in aliquots of the above incubations by the manometric method of WINZLER[6] employing *S. cerevisiae* in the presence of sodium azide. The yeast used was obtained by repeated subculture of a strain isolated from commercial baker's yeast. Specific enzyme activity is here expressed in units corresponding to the number of milligrams of lactose hydrolysed per milligram of protein under the incubation conditions described. The enzyme was rate limiting in the system until at least 10% hydrolysis of the substrate had occurred.

* This work was supported by a research grant from the National Institutes of Health, Public Health Service.

** Present address — Department of Biochemistry, Cornell University, Ithaca, New York.

References p. 286.

RESULTS

Purification procedure

The mucosae from two to four calves were worked up as follows: After thawing, the intestines were split lengthwise and the mucosa scraped from the outer membrane.

1. *Autolysis.* The gelatinous mass (approximately three liters) was then mixed with about 30 ml of toluene per liter of mucosa and allowed to autolyse for two days at room temperature. Celite 535, filter aid (Johns-Manville), was added with vigorous shaking until the mass remained in one lump and failed to stick to the walls of the flask. An equal volume of water was then mixed in and the solids allowed to settle. The supernatant fluid was poured off and filtered under pressure through a thin layer of the filter aid. The lower layer was likewise filtered, the solid material extracted with one liter of water and the extract filtered under pressure. About three liters of faintly turbid reddish-coloured filtrate containing approximately 60 g of protein, of specific activity 0.70 to 1.0 units, were obtained. All subsequent operations were carried out in a 0° C cold room.

2. *Ammonium sulfate fractionation.* The liquid was made 0.05 *M* with respect to phosphate ion, about two grams of lactose were added and the pH adjusted to 6.7 with sodium hydroxide. Solid ammonium sulfate was added with mechanical stirring to 0.45 saturation with respect to the salt (318 g salt per liter of protein solution). The pH was maintained at 6.7 by addition of sodium hydroxide. After stirring for one half hour the precipitate was removed by pressure filtration through Celite 535 supported by Nylon filter cloth. The solid was discarded. The ammonium sulfate concentration of the filtrate was raised to 0.68 saturation at pH 6.7 and the solid separated as above. The red precipitate was dissolved from the filter with successive portions of water containing 0.5% lactose. The 400 milliliters of solution so obtained contained about 70% of the lactase of the autolysis filtrate and the activity was 2.0 to 3.0 units.

3. *Dialysis and lyophilization.* The ammonium sulfate fraction was dialysed against two or three changes of 0.10 *M* phthalate buffer at pH 5.0, and then against four or five changes of water. Variable amounts of inactive protein were precipitated by this procedure. Dialysis, against water only, resulted in considerable loss of lactase.

After removal of the inactive precipitate by centrifugation the solution was lyophilized to yield about 12 g of buff-coloured protein whose specific activity was in the range of 2.5 to 5.0 units. The dried material was stable when stored in the cold. No loss of activity ensued from the drying operation.

The autolysis filtrates from fetal calf intestines were found to have specific activities two to three times greater than those from calf intestines. This superiority was maintained through the freeze-drying step, *i.e.*, the dried material from step 2 had average specific activity of about 9.5 units. No fetal preparations were carried beyond this stage of purification.

4. *Ethanol Fractionation.* Considerable purification was achieved by fractionation with alcohol owing to denaturation and consequent lower water solubility of extraneous protein. Twelve grams of the dried protein were dissolved in 240 ml of 1.0 *M* sodium acetate of pH 5.0. Precooled absolute ethanol was added through a capillary to the stirred solution to a concentration of 50% by volume. At the start, the temperature of the protein solution was 0°. During addition of the alcohol the temperature was lowered with an ice-salt bath to a final value of —15° C as rapidly as possible without

References p. 286.

freezing. Stirring was continued for one-half hour and the bulky inactive precipitate removed by centrifugation at —10° C. Sufficient alcohol calculated to raise the volume concentration of the original extract to 60% was then added as before, the volume of the first precipitate being neglected. The resulting precipitate containing most of the lactase was removed as previously. The solid was then extracted by stirring thoroughly with as small a volume of water as feasible (two to four milliliters). After centrifugation at 0° C for one-half hour at 3000 r.p.m. (Size I International Centrifuge) the clear upper layer containing the enzyme was poured off. The yield for the fractionation was about 55% and could be raised to 70% by a second extraction of the solid material. The specific activity of the first extraction ranged from 70 to 100 units, the second extraction being somewhat lower. Both activity and yield in this step appeared to depend on the volumes used for the extractions, presumably owing to the small but definite solubility of the denatured extraneous protein. This was evidenced in some cases by a slight colour in the extracts, presumably derived from the chocolate-coloured residue. The above described procedure was established after trials with phosphate, ammonium acetate, and sodium acetate buffers in the pH range 5.0 to 7.7 and with ionic strengths as low as 0.01. These most active preparations were not stable to freeze-drying and storage at 0° C.

Bovine intestinal phosphatase (Armour) was found to exhibit specific lactase activity of about 1.0 unit, *i.e.*, similar magnitude to the autolysis filtrate described here.

Twenty-five grams were dissolved in water, then dialysed, lyophilized and precipitated with ethanol as described in 4. Extraction of the protein fraction appearing between 50 and 60% concentration by volume of alcohol resulted in peptization so that water soluble and insoluble portions could not be obtained by ordinary centrifugation. The specific activity of this opalescent liquid was about ten units and the overall recovery 20%.

Characteristics of intestinal lactase

Optimum pH. Maximum activity of the lactase was exhibited between pH 5.5 and 6.0. This range was unaffected by the purity of the enzyme or the type of buffer salts used. Citrate, phosphate, phthalate, and maleate, were used in appropriate ranges.

Stability of lactase

Stability at various hydrogen-ion concentrations was measured by buffering portions of a solution of purified lactase at various pH values between 3.5 and 8.0 and allowing the samples to remain for one to two hours at 0°. Maximum stability was exhibited at pH 5.0, dropping nearly 50% at the ends of the range tested.

Influence of other substances. Glucose added in one-half the initial concentration of lactose caused 70% inhibition of hydrolytic activity. Galactose added in concentration equal to that of lactose had no effect. This is in accordance with the finding of CAJORI[3] for dog intestinal lactase.

No influence on activity was noted when the following ions were added in 0.02 *M* concentration: SO_4^{-2}, Br^{-1}, Cl^{-1}, NO_3^{-1}, I^{-1}, CNS^{-1}, PO_4^{-3}, CH_3COO^-, NH_4^{+1}, Mg^{+2}, Ca^{+2}, Ba^{+2}. Some inhibition occurred in the presence of 0.10 m*M* Hg^{+2} or Cu^{+2}. Adequate quantitative data were not obtained regarding this inhibition, since lower than usual blanks were obtained in the yeast-glucose assay system.

Other enzyme activities in lactase extracts

A lactase preparation with a specific activity of 60 units was tested for acid and

References p. 286.

alkaline phosphatase, as well as for invertase activity. Phosphatase assays were done as described by HAWK, OSER, AND SUMMERSON[7]. At pH 5.0, 0.40 mg of lactase protein hydrolysed 0.45% of the glycerophosphate; at pH 9.0 the same amount of protein split 2.90% of the substrate.

No invertase activity was observed polarimetrically after incubation of 0.5 mg of lactase for one hour at 30° C in 4.0 ml of phosphate buffer, pH 7.0, containing 100 mg of sucrose.

DISCUSSION

The purification procedures reported are capable of purifying intestinal lactase by a factor of 100 from an autolysis filtrate in good yield. This undoubtedly represents a minimum value calculated from the actual starting material, which could not be accurately assayed because of its gel-like characteristics and the presence of particulate matter.

One of the purified preparations was revealed to be still impure by electrophoretic analysis.

Rough calculations based on wet weight of tissue, indicate that the concentration of lactase in calf-intestinal mucosa is of the same order as that found in mucosa of dogs.

An average of values reported by CAJORI[3] shows hydrolysis of 26 mg of lactose per gram of tissue per hour; calculations in this work from the activity of the autolysis filtrate back to the weight of original tissue averaged near 20 mg of substrate split per g of tissue per hour, assuming complete recovery of the enzyme in the autolysis filtrate. The enzyme appears to be extracted more easily from calf mucosa than from dog mucosa.

ACKNOWLEDGEMENTS

The authors are indebted to Dr E. E. HAYS and Dr J. B. LESH of Armour and Company for generous samples of intestinal phosphatase, and to Dr J. C. NICHOL, Willamette University, Salem, Oregon for the electrophoretic analysis.

SUMMARY

A procedure for preparing a purified lactase from calf intestine is described. The enzyme preparation obtained has about 100 times the activity of the original extract, an optimum pH range of 5.5–6.0, and is maximally stable in solution at pH 5.0. Glucose inhibits hydrolytic action of the enzyme but galactose has no effect.

RÉSUMÉ

L'auteur décrit la préparation d'une lactase purifiée à partir de l'intestin de veau.

L'activité de l'extrait purifié est environ cent fois plus élevée que celle de l'extrait original. L'enzyme montre un maximum d'activité dans le domaine de pH 5.5–6.0 et un maximum de stabilité en solution à pH 5.0. L'action hydrolytique est diminuée en présence de glucose, tandis que la galactose n'a aucun effet.

ZUSAMMENFASSUNG

Es wird ein Verfahren zur Darstellung einer gereinigten Laktase aus Kalbsdarm beschrieben. Das hierdurch gewonnene Fermentpräparat zeigt eine etwa verhundertfachte Aktivität im Verhältnis zum Ausgangsstoff, mit optimalem pH zwischen 5.5 und 6.0. In Lösungen hat es seine maximale Stabilität bei pH 5.0. Glukose hemmt die hydrolytische Wirkung des Ferments, Galaktose dagegen nicht.

REFERENCES

1 M. G. HOROWITZ, H. M. DAVIDSON, F. D. HOWARD, AND F. J. REITHEL, *Anal. Chem.*, 23 (1951) 375.
2 R. H. A. PLIMMER, *J. Physiol.*, 35 (1906) 20.
3 F. A. CAJORI, *J. Biol. Chem.*, 109 (1935) 159.
4 A. G. GORNALL, C. J. BARDAWILL, AND M. M. DAVID, *J. Biol. Chem.*, 177 (1948) 751.
5 T. BUCHER, *Biochim. Biophys. Acta*, 1 (1947) 292.
6 R. WINZLER, *Science*, 99 (1944) 327.
7 P. B. HAWK, B. L. OSER, AND W. L. SUMMERSON, *Practical Physiological Chemistry*, p. 584, Blakiston 1947.

Received January 9th, 1952