BBA 66602

ENZYMIC RELEASE OF ENTEROPEPTIDASE FROM ISOLATED RAT DUODENAL BRUSH BORDERS

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(Received December 20th, 1971)

SUMMARY

1. The release of enteropeptidase (EC 3.4.4.8), formerly known as enterokinase, from isolated rat duodenal brush borders by various enzymes (especially pancreatic proteases) was studied and compared with the release of other enzymic activities present in the brush border (lactase (EC 3.2.1.23), sucrase (EC 3.2.1.26), isomaltase, maltase (EC 3.2.1.20), trehalase (EC 3.2.1.28), alkaline phosphatase (EC 3.1.3.1) and leucyl naphthylamidase). A study on the release by bile salts was included for direct comparison.

2. The enterokinase activity was released more easily by papain (EC 3.4.4.10) than any of the other enzymic activities. Trypsin (EC 3.4.4.4) and chymotrypsin (EC 3.4.4.5) both caused a significant release of enterokinase activity but no or negligible release of the other enzymic activities. Carboxypeptidase and hyaluronidase did not release any brush-border enzymes. Bile salts released enterokinase activity but also other enzymic activities in equivalent amounts.

3. The results indicate that trypsin and chymotrypsin may contribute to create the considerable enterokinase activity present in the duodenal juice. They are also in accord with the concept that enterokinase is superficially located in the brush-border membrane and that the brush-border enzymes are organized within the membrane in a mosaic-like pattern.

INTRODUCTION

Enteropeptidase (EC 3.4.4.8), formerly known as enterokinase, has long been regarded as a secretion product from the deep layers of the small-intestinal mucosa. Recently we found, however, that this enzyme is located in the villi and bound to the superficial brush-border membrane of the villous epithelial cells[1–4], which does not seem to support the concept of its secretion into the lumen. Nevertheless, considerable enterokinase activity can be demonstrated in human duodenal juice after pancreozymin stimulation, indicating that the anatomical and functional localization of enterokinase are perhaps not identical[5]. The activity of enterokinase in the lumen is proportionally larger (compared with the activity of the mucosa) than the activity

of such other brush-border enzymes as sucrase (EC 3.2.1.26) and maltase (EC 3.2.1.20) both in the fasting state and after a test-meal (unpublished work in collaboration with A. Dahlqvist and Å. Nordén).

One factor certainly contributing to the lumenal enterokinase activity is physiological cell desquamation, which also seems capable of causing differences in the lumenal levels of the various brush-border enzymes[4]. However, cell shedding alone seems to not fully explain the presence of large amounts of soluble enterokinase activity in the intestinal juice; some other mechanism (mechanisms) seems to operate in addition. Pavlov[6] stated long ago that enterokinase is secreted from the intestinal wall on direct stimulation by pancreatic proteases. More recently, Lepkovsky *et al.*[7] arrived to the same conclusion after studies on the lumenal enterokinase activity of chickens in the presence and absence of active pancreatic proteases. They make no comments on their findings in relation to the localization of enterokinase in the intestinal wall, which was not known to them. It is apparent, however, that a direct action of lumenal factors on the enterokinase activity of the intestine may be anatomically possible owing to the superficial cellular and subcellular localization of the enzyme[4].

To elucidate one type of factors involved, the present author has performed a study on the release of enterokinase and other brush-border enzymes from isolated rat brush borders by various enzymes, especially pancreatic proteases. It was also anticipated that such an enzymic dissection might contribute to our understanding of the morpho–chemical relationship between enterokinase and other brush-border enzymes and between enterokinase and the membrane[8].

Recently, Hadorn *et al.*[5] reported that bile salts may be responsible for a physiological release of enterokinase from the intact cells of the villi, as judged from experiments with isolated rat brush borders. Their experiments have been repeated here to allow a direct comparison with the effect of proteases.

Preliminary results have recently been published in the form of a letter[9].

MATERIAL AND METHODS

Isolation of brush borders

Rat duodenal brush borders were obtained as previously described[4]. The animals (Wistar strain) were starved for 20–24 h before use. Mucosal scrapings from duodenum and a few cm of adjacent jejunum only were used for two reasons: first, the duodenum is the part of the intestine to which the trypsinogen of the pancreatic juice is presented; secondly, the highest activity of enterokinase has been found in duodenum[4]. The crude brush border suspensions prepared according to Miller and Crane[10] were purified by NaCl treatment[11] before use. The specific activity of the enterokinase and sucrase was about 10 times higher in the purified brush border preparation than in the original homogenate. Some authors have reported a much higher degree of purification of the enterokinase than of the sucrase activity[5,12], but this is not in agreement with our experience.

Chemicals

α-Chymotrypsin (EC 3.4.4.5) (3 × crystallized; lyophilized) and carboxypeptidase (EC 3.4.2.1) (3 × crystallized; suspension) from beef pancreas and papain

(EC 3.4.4.10) (2 × crystallized; suspension) were purchased from NBC, Cleveland, Ohio, U.S.A.

Crystallized trypsin (EC 3.4.4.4) from bovine pancreas was obtained from Ferrosan, Malmö, Sweden.

Sodium taurocholate and sodium taurodeoxycholate were kindly supplied by Professor B. Borgström and Dr. B. Arnesjö, Lund, Sweden.

Incubation procedure

The isolated brush borders were incubated at 25 °C in 14.3 mM potassium phosphate buffer (pH 7.1) with 1.8 mM sodium EDTA in the presence of the different enzymes and bile salts specified above. The concentration of brush-border protein was between 0.2 and 0.4 mg/ml incubation mixture in all experiments.

After incubation, the test tubes were immediately chilled with ice-water, and the brush borders were sedimented by centrifugation at 30 000 × *g* for 30 min at 4 °C. The supernatant (released fraction) and the resuspended sediment were analyzed for enterokinase and other enzymic activities of the brush border.

When the effect of papain was tested, the enzyme was first activated by cysteine; cysteine was also present in the incubation mixture (2.5 mM).

When trypsin was used, the released enterokinase activity could not be assayed in the supernatant, since this contained an excess of trypsin. The release of enterokinase was in these experiments calculated only from the decrease in activity in the sediment. The sediment was washed once to remove trypsin before the final resuspension. In these experiments it was also not possible to calculate the total recovery of enterokinase activity.

Enzymic assays

Enterokinase activity was determined by a method previously described in detail[4]. Bovine trypsinogen was used as the substrate. The amount of trypsin formed was estimated in a subsequent step with the chromogenic method of Erlanger *et al.*[13] using benzoyl DL-arginine *p*-nitroanilide·HCl as the substrate.

Disaccharidase activities were measured with the two-step Tris–glucose oxidase method of Dahlqvist[14].

Alkaline phosphatase (EC 3.1.3.1) activity was determined with a modification of the method of Bessey *et al.*[15] using *p*-nitrophenyl phosphate as substrate, in the presence of magnesium, zinc and cobalt ions as activators[16].

Leucyl naphthylamidase activity was measured by the fluorimetric method of Uete *et al.*[17], except that the reaction mixture was made 1 mM with respect to cobalt ions.

Protein was estimated by the method of Lowry *et al.*[18] as modified by Eggstein and Kreutz[19].

RESULTS

Release by papain

The release of various enzymic activities from isolated rat brush borders by treatment with papain is seen in Fig. 1. The enterokinase activity was released much more rapidly than any of the other activities studied. About half of the total entero-

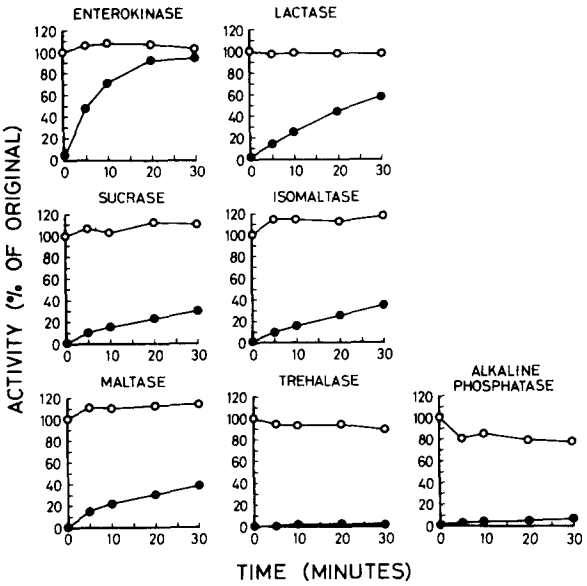

Fig. 1. Release of enzymic activities from isolated rat brush borders with time of treatment with papain (1.3 μg/ml incubation mixture). ●, the released fraction at various times; ○, total activity (recovery) at the corresponding times. Values in the curves have in both cases been expressed as per cent of the total activity present at zero time.

kinase activity was liberated during conditions which released only small amounts of the other brush-border enzymes. The release curves also showed clear differences between these other enzymes. The lactase (EC 3.2.1.23) activity was more easily solubilized than the other disaccharidases. The sucrase, isomaltase and maltase activities had similar release curves, whereas the trehalase (EC 3.2.1.28) activity was not released at all. Alkaline phosphatase and leucyl naphthylamidase (not illustrated) were liberated to a very small extent.

The solubilization of enterokinase by papain was not associated with any significant increase in the activity of the enzyme as judged from the recovery values (Fig. 1).

Examination of the incubation mixture at high resolution in the phase contrast microscope during incubation with papain (Fig. 1) showed that the brush borders gradually disintegrated. In the beginning of the incubation, however, when already significant amounts of enterokinase had been released, the structure of the brush borders still seemed to be rather intact.

Release by chymotrypsin

Chymotrypsin (0.14 mg/ml) caused a still more specific release of the enterokinase activity than papain (Fig. 2). The release of 50–60% of the enterokinase activity was accompanied by release of only small amounts of lactase and no or negligible release of other enzyme activities. Lower concentrations of chymotrypsin (0.07 and 0.1 mg/ml) were usually still as effective as 0.14 mg/ml in liberating enterokinase. Complete release of the enterokinase activity was not even seen at very high

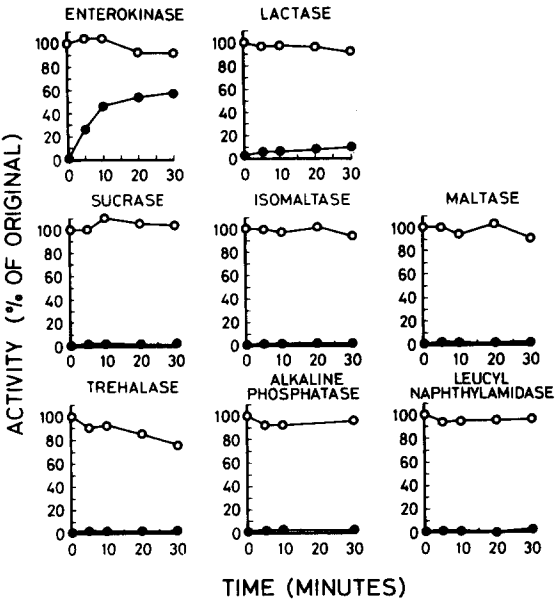

Fig. 2. Release of enzymic activities from isolated rat brush borders with time of treatment with chymotrypsin (0.14 mg/ml incubation mixture). For the explanation of the curve symbols see Fig. 1.

protease concentrations (2.9 mg/ml). At all concentrations of chymotrypsin the other brush border enzymes were released in very limited amounts.

During the incubation (Fig. 2), a progressive fragmentation of the brush borders occurred. However, in the first minutes—corresponding to the most rapid release of enterokinase activity—no great changes in the morphology of the brush borders were seen.

Release by trypsin

Trypsin gave a release pattern very similar to that caused by chymotrypsin. The enterokinase activity was significantly released, whereas other enzymic activities were not released or released to a very small extent. 30–55% of the total enterokinase activity was released in 25 min by trypsin in a concentration of 0.06 mg/ml incubation mixture and the released fraction remained unaltered when the amount of trypsin was increased up to 20-fold.

Microscopic examination of brush borders during treatment with trypsin revealed that they gradually disintegrated.

Although trypsin and chymotrypsin did not significantly release any enzymic activities from isolated brush borders except for enterokinase (chymotrypsin also liberated small amounts of lactase activity), they were also able to release different disaccharidase activities (except trehalase) from the membrane fragments of a mucosal homogenate. For instance, 48% and 34% of the particle-bound sucrase activity present in a diluted homogenate (0.9 mg protein/ml incubation mixture) were released in 25 min at 25 °C by trypsin and chymotrypsin (1 mg/ml incubation mixture), respectively. Other experimental conditions were all identical to those in

the experiments with isolated brush borders. The homogenate was prepared by Ultra-Turrax homogenization for 2 min (4 ml water/g mucosa). This vigorous, mechanical treatment is known to disintegrate the surface membrane of the digestive cells into very small fragments[20]. It also caused a mechanical solubilization of 10–15% of the sucrase activity.

Release by other enzymes

Carboxypeptidase and hyaluronidase did not cause release of either enterokinase or other brush-border enzymes.

Release by bile salts

Incubation with 9.4 μmoles bile salts per ml incubation mixture (sodium taurocholate and sodium taurode oxycholate 2/1) for 20 min at 25 °C solubilized about 35% of the enterokinase activity, but also released other brush-border enzymic activities to at least the same extent. The solubilization step as such did not cause any significant increase in the activity of enterokinase. Microscopic examination of the brush borders during incubation with bile salts revealed that a disintegration occurred.

The sedimentation properties of bile-salt-solubilized enterokinase activity were studied after removal of the bile salts by dialysis or ethanol precipitation. The purpose was to investigate if this form of the enzyme is truly soluble or if its retention in the supernatant is due to the presence of bile salts. It was found that the major part of the activity was possible to sediment (100 000 $\times$ g for 60 min) after dialysis for 65 h against 5 mM sodium maleate buffer (pH 6.0). The readdition of bile salts prior to the centrifugation prevented most of this sedimentation. When bile-salt-solubilized enterokinase activity was precipitated with cold ethanol and resuspended in buffer only, the major part of the enterokinase activity sedimented in an ordinary laboratory centrifuge (5000 rev./min for 20 min).

The important finding by Hadorn *et al.*[5] that the activity of enterokinase (isolated brush borders) is about five times increased in the presence of an optimal concentration of bile salts was verified. The same increase was also found for both bile-salt-solubilized and protease-solubilized enterokinase activity. Furthermore, the enterokinase activity remaining in the sediment (bile-salt solubilization was not 100%) was activated.

DISCUSSION

The results indicate that trypsin and chymotrypsin may contribute to create the considerable amounts of soluble enterokinase activity present in the lumen. Both proteases caused a release in concentrations well below, as well as around, physiological. Their proteolytic action on the surface membrane of intact and/or predesquamated cells of the villi could explain why the duodenal juice contains more enterokinase than disaccharidase activity, and it could also account for the soluble form of enterokinase present in the juice. The fact that trypsinogen must be activated by enterokinase (and chymotrypsinogen by trypsin) before the proteases become active is no argument against such a mechanism, since the trypsinogen is partly activated by enterokinase while this enzyme is still associated with the intestinal wall

(unpublished observation). Furthermore, enterokinase is liberated from the mucosa in desquamated cells[4], which, after disintegration, will make the enterokinase as accessible as in the isolated brush borders. Experiments using more physiological methods are needed, however, before the importance of pancreatic proteases for the lumenal enterokinase activity in vivo can be finally evaluated.

The enzymic release of enterokinase may have both a chemical and a structural explanation. It is apparent that the linkages of enterokinase to the surface membrane may differ chemically from those of the other enzymes, being more sensitive to the different proteases. It should be noted, however, that the specificities of papain, trypsin and chymotrypsin are not identical. The findings on the release would also be in good accord with a very superficial location of enterokinase in the surface membrane, possibly even in the glycocalyx. A superficial site in the membrane of this enzymic activity is also indicated by the fact that the proteolytic solubilization of enterokinase of isolated brush borders was not associated with any increase in the efficiency of trypsinogen activation, although this substrate is a macro-molecule. Furthermore, trypsin and chymotrypsin released almost solely enterokinase from isolated brush borders. When these proteases were incubated with a homogenate prepared in a way that disintegrates the brush borders into small fragments, however, they were able to solubilize both enterokinase and a number of disaccharidases. Thus, in the intact brush border enterokinase is more accessible to the proteases than are the disaccharidases. This indicates ultrastructural differences in the localization between enterokinase and for instance sucrase, probably with the site of enterokinase more close to the surface of the membrane.

It has been proposed that the enzymic activities within the brush-border membrane are not diffusely distributed throughout the protein coat of the membrane but organized in discrete packets, in morphochemical subunits as in a mosaic[8,21,22]. The present results on the enzymic release of enterokinase represent a new indication for the correctness of this concept.

The experiments with bile salts confirm the recent observation by Hadorn *et al.*[5] that bile salts solubilize part of the enterokinase activity present in isolated brush borders. It can be questioned, however, if it is a true solubilization or whether it is rather mainly a formation of low-specific-weight aggregates with relatively large membrane fragments as has been observed for other brush border enzymes[20]. Two observations seem to support the latter possibility: first, bile salts solubilize all brush-border enzymes in equivalent amounts; secondly, when bile-salt-solubilized enterokinase activity was centrifuged after removal of the bile salts by dialysis or ethanol precipitation, the major part of the activity behaved as if it was particulate rather than soluble.

The enterokinase activity of isolated brush borders increased up to about five times original in the presence of increasing concentrations of bile salts. This increase was not due to the solubilization of enterokinase activity as stated by Hadorn *et al.*[5], but was found to be due to the ability of bile salts to activate the enterokinase activity irrespective of whether the activity is present in soluble or bound form.

ACKNOWLEDGEMENTS

Thanks are due to Dr Arne Dahlqvist for valuable discussions and criticism.

The investigation was supported by grants from the Medical Faculty of the University of Lund, The Swedish Nutrition Foundation and the Swedish Medical Research Council (grant to A.D. project No. B71-13X-157 and K71-13P-2262).

Miss Ulla Iwarson is thanked for skilful technical assistance.

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