Putative tripeptidyl peptidase in renal brush border is due to sequential action of two other exopeptidases

To the Editor: We wish to draw readers' attention to the need for caution in interpreting assays of peptidases using fluorogenic substrates such as Gly-Pro-Met-2-naphthylamide (NNap). The release of the fluorophore, 2-naphthylamine, may indicate the action of a tripeptidyl peptidase releasing the tripeptide, Gly-Pro-Met, but it may also result from the sequential attack of other exopeptidases, first releasing Gly-Pro and then free methionine. The essential criterion of a tripeptidyl peptidase attack is the demonstration of the tripeptide among the products of the reaction.

Two recent reports by Knut-Jan Andersen and J. Ken McDonald (1, 2) of a tripeptidyl peptidase active at pH 7 in kidney brush-border membranes do not contain any evidence that a tripeptide was released from the naphthylamide substrate. We have now reinvestigated this question and fail to find a tripeptidyl peptidase hydrolyzing either Gly-Pro-Met-NNap or Gly-Pro-Leu-NNap in pig or rat microvillar membranes (3). The hydrolysis of both substrates was readily assayed fluorimetrically, but the products analyzed by high precision liquid chromatography were Gly-Pro, Met-NNap (or Leu-NNap) and free naphthylamine, with no evidence of tripeptidases. Moreover, the activity monitored by the assay was inhibited by either diisopropylfluorophosphate or amastatin (inhibitors of dipeptidyl peptidase IV and aminopeptidase N, respectively) and could be titrated to zero by polyclonal antibodies raised to pig and rat aminopeptidase N. It was our conclusion that these substrates were hydrolyzed by renal microvilli in a two-step process, first the release of Gly-Pro by dipeptidyl peptidase IV and then the hydrolysis of the amino acid naphthylamide by aminopeptidase N. There are twelve well-characterized peptidases so far identified in renal microvilli (4), but a true tripeptidyl peptidase cannot yet be added to the list.

REFERENCES

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REPLY

To the Editor: The letter submitted by A. John Kenny and Jean Ingram cautions readers of the possible misinterpretation of fluorometric tripeptidyl peptidase (TPP) assays that employ tripeptidyl-2-naphthylamide (NNap) substrates such as Gly-Pro-Met-NNap. They have addressed a potential analytical problem of the sort that has long been a source of concern to those involved in the development of fluorogenic substrates for the specific assay of dipeptidyl- and tripeptide-releasing exopeptidases.

The misinterpretation of fluorometric TPP assays is especially likely if kinetic aspects of the reaction are not considered. A reaction rate (which reflects the rate of formation of free 2-naphthylamine) that is initially linear is generally indicative of a direct attack on the arylamide bond, and consequently is likely to reflect a genuine TPP activity. (Fluorometric TPP assays that employ direct, continuous rate measurements should therefore be based on the initial slope of the tracing.) In contrast to a TPP-catalyzed reaction, the sequential breakdown of a tripeptidyl-NNap by the concerted action of other exopeptidases would be expected to display an initial lag followed by an accelerating rate of fluorescence increase. In our first report (1), which described evidence for the possible presence of a tripeptidyl peptidase active at pH 7.0 ("TPP 7") in renal brush-border membranes, we reported that assessments of the reaction kinetics and specificity revealed a linear rate of hydrolysis on Gly-Pro-Met-NNap and a lack of action on succinyl-Gly-Pro-Met-NNap. The latter observation, which demonstrated a requirement for an unsubstituted N terminus, was consistent with the action of a putative tripeptidyl peptidase, and ruled out the possible contribution of an endopeptidase to the direct hydrolysis of the arylamide bond.

As Kenny and Ingram (3) have indicated, proof of a tripeptidyl peptidase attack requires the demonstration of a tripeptide product. However, when one is working with crude preparations, as we were, such an undertaking is usually unrewarding since liberated peptides are susceptible to attack by endogenous peptidases. Such interfering activities contributed to the difficulties experienced by Kenny and Ingram (3) in their unsuccessful attempts to demonstrate a tripeptide product in Gly-Pro-Met-NNap digests that employed microvillar membranes, with their host of associated peptidases. Inhibitors such as diisopropylfluorophosphate (DIP-F) and amastatin were employed in an attempt to block selectively the action of prominent complicating exopeptidase activities present in the brush-border membrane. Hazards arise, however, from the use of such inhibitors because the only two tripeptidyl peptidases thus far characterized are sensitive to DIP-F (2, 4). The effect of amastatin, a modified tripeptide, on TPP II (the exopeptidase thought to be responsible for the TPP activity...
we reported) was not included among the inhibitors investigated by Bålow et al. (2). Even if intact Gly-Pro-Met were produced in the presence of these inhibitors, it would probably be vulnerable to attack by another member of the microvillar complex, namely carboxypeptidase P (microsomal prolyl carboxypeptidase), a metalloprolyl carboxypeptidase unaffected by the inhibitors employed (4).

In any comparison of experimental conditions it should be noted that the rat kidney subcellular fractions employed in our study differed greatly from the purified brush-border membranes employed by Kenny and Ingram (3). The activities contained in our fractions were derived from rat renal cortical homogenates by differential pelleting. Fractions studied were prepared by the subfractionation of microsomes or lysosomes/microsomes by rate sedimentation and isopycnic banding. Purified organelles were not generated, but rather fractions enriched in a particular organelle. Accordingly, since our claim of a possible brush-border localization for the TPP 7 activity was based solely on distribution profiles that resembled those displayed by commonly used brush-border marker enzymes, the reader was appropriately cautioned regarding the heterogeneity of the fractions employed.

Although the rat kidney has been shown (by means of an unambiguous assay) to be a relatively rich source of an extralysosomal tripeptidyl peptidase (TPP II) active at neutral pH (2), we agree with Kenny and Ingram (3) that an unequivocal demonstration of such an enzyme in renal microvilli has yet to be furnished.

REFERENCES


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