ε-(γ-Glutamyl)lysine: Hydrolysis by γ-Glutamyltransferase of Different Origins, When Free or Protein Bound

Katsuya Seguro,* Yoshiyuki Kumazawa, Tomoko Ohtsuka, Hiroyuki Ide, Noriki Nio, Masao Motoki, and Koji Kubota

Food Research and Development Laboratories, Ajinomoto Company, Inc., 1-1 Suzuki-cho, Kawasaki-ku, Kawasaki-shi 210, Japan

ε-(γ-Glutamyl)lysine, a moiety found in various tissues, organs, and processed foods, is the product of either intrinsic transglutaminase reaction or chemical reaction during cooking processes. From a nutritional viewpoint, hydrolysis of the ε-(γ-glutamyl)lysine bond and bioavailability of its constituent lysine have been the targets of investigation. In this study, cleavage of the ε-(γ-glutamyl)-lysine bond and liberation of free lysine and glutamic acid by γ-glutamyltranspeptidase (γ-GTP) from bovine kidney were found. Also, after exhaustive hydrolysis of transglutaminase-catalyzed cross-linked minced fish paste ("surimi") protein, undigested ε-(γ-glutamyl)lysine moiety in the hydrolysate by current gastrointestinal hydrolytic enzymes was cleaved by γ-GTP. These results suggest that during the gastrointestinal digestion of the cross-linked proteins, γ-GTP in the intestinal mucosal wall would hydrolyze and lysine in the proteins would be utilized normally.

Keywords: *γ-Glutamyltransferase; γ-glutamyltranspeptidase; ε-(γ-glutamyl)lysine; transglutaminase*

INTRODUCTION

ε-(γ-Glutamyl)lysine [ε-(γ-Glu)Lys] cross-link has been found in various tissues and organs of mammals, plants, fish eggs, and microorganisms and proven to be mostly the enzymatic product of endogenous transglutaminase (glutamyl-peptide γ-glutamyltransferase, TGase; EC 2.3.13.2) (Folk, 1983; Folk and Chung, 1973; Hagenmaier et al., 1976; Folk and Finlayson, 1977; Loewy, 1984). Formation of ε-(γ-Glu)Lys cross-links can also result in textural changes of many protein-rich products, and such enzymatic activity has been utilized in the creation of new materials (Nio et al., 1986; Motoki et al., 1987; Kato et al., 1991; Nonaka et al., 1992, 1994). The cross-linking moiety, ε-(γ-Glu)Lys, is practically resistant to hydrolysis by ordinary gastrointestinal proteolytic enzymes, and thus exhaustive proteolysis *in vitro* of the cross-linked protein products by such enzymes yields an indigestible ε-(γ-Glu)Lys dipeptide from the proteins. This method has been applied in the analysis of ε-(γ-Glu)Lys in various proteins and TGase-treated foods (Griffin et al., 1982, 1984; Sato et al., 1992; Kumazawa et al., 1993).

The digestibility of the ε-(γ-Glu)Lys bond and the bioavailability of its constituent lysine have come into question. As a solution to this, an enzyme responsible for digestion of ε-(γ-L-Glu)-L-Lys *in vivo*, γ-glutamylamine cyclotransferase, was reported (Fink et al., 1980). This enzyme spreads ε-(γ-L-Glu)-L-Lys dipeptide into lysine and 5-oxo-L-proline (synonyms: pyroglutamic acid, 5-pyrrolidone-2-carboxylic acid). On the other hand, γ-glutamyltranspeptidase (γ-GTP; EC 2.3.2.2), a membrane-bound enzyme, has the ability to catalyze transfer reaction of the γ-glutamyl moiety in glutathione, S-substituted glutathione derivatives, and other γ-glutamyl compounds to a number of acceptors. When the acceptor is an amino acid (or a dipeptide), a γ-glutamyl amino acid (or a γ-glutamyl dipeptide) is formed. If the γ-glutamyl donor is also the acceptor, autotranspeptidation occurs. When the acceptor is water, the overall result is hydrolysis (Meister et al., 1981).

In this manner, the γ-glutamyl linkage in ε-(γ-Glu)-Lys was thought to be cleaved by γ-GTP. Thus, degradation of ε-(γ-Glu)Lys dipeptide itself and of the ε-(γ-Glu)Lys moiety in TGase-catalyzed cross-linked surimi protein by γ-GTP were investigated in this study. The nutritional value of ε-(γ-Glu)Lys as a source of the essential amino acid lysine is discussed.

MATERIALS AND METHODS

Chemicals. Tris(aminohydroxy)methane, *o*-phthalaldehyde (OPA), common amino acids, methanol, and acetonitrile were of guaranteed grade and purchased from Wako Pure Chemical Co. (Osaka, Japan). Methanol and tetrahydrofuran (THF) for the liquid chromatography were of liquid chromatography grade. ε-(γ-Glu)Lys dipeptide was a product of Sigma Chemical Co. (St. Louis, MO).

Enzymes. Microbial transglutaminase (MTGase, EC 2.3.2.13) was obtained as described previously from the cultural broth of a nontoxicogenic variant of *Streptoverticillium mobaraense* (Ando et al., 1989; Washizu et al., 1994). γ-Glutamyltranspeptidase (EC 2.3.2.2) from bovine kidney was purchased from Wako Pure Chemical, and γ-glutamyltranspeptidases from equine and porcine kidney were purchased from Sigma. They were used without further purification. Microbial γ-glutamyltranspeptidase from *Escherichia coli* K-12 was a gift from Dr. Kumagai, Kyoto University. Pronase, leucine aminopeptidase, prolidase, and carboxypeptidase A used for exhaustive digestion were purchased from Boehringer Mannheim Yamanouchi Co. (Tokyo, Japan) and Sigma Chemical Co.

Preparation of Cross-Linked Minced Fish ("Surimi") Protein. Two hundred and fifty grams of frozen minced fish paste ("surimi", SA grade, Maruha Co., Tokyo, Japan) was chopped in a speed cutter (Model MK-K3, Matsushita Electric Industrial Co. Ltd., Japan) for 5 min, followed by chopping with salt (3% w/w), water (20% w/w), and microbial TGase (0.07% w/w) for 4 min. The surimi paste was stuffed into 30-mm diameter poly(vinylidene chloride) tubes, and then the tubes were sealed and immersed in a water bath (45 °C) for 30 min. After the incubation, the paste turned to gels and was cooked at 85 °C for 30 min and cooled in ice.

* Author to whom correspondence should be addressed (fax +81-44-244-7198; e-mail nio@mi.staff.or.jp).

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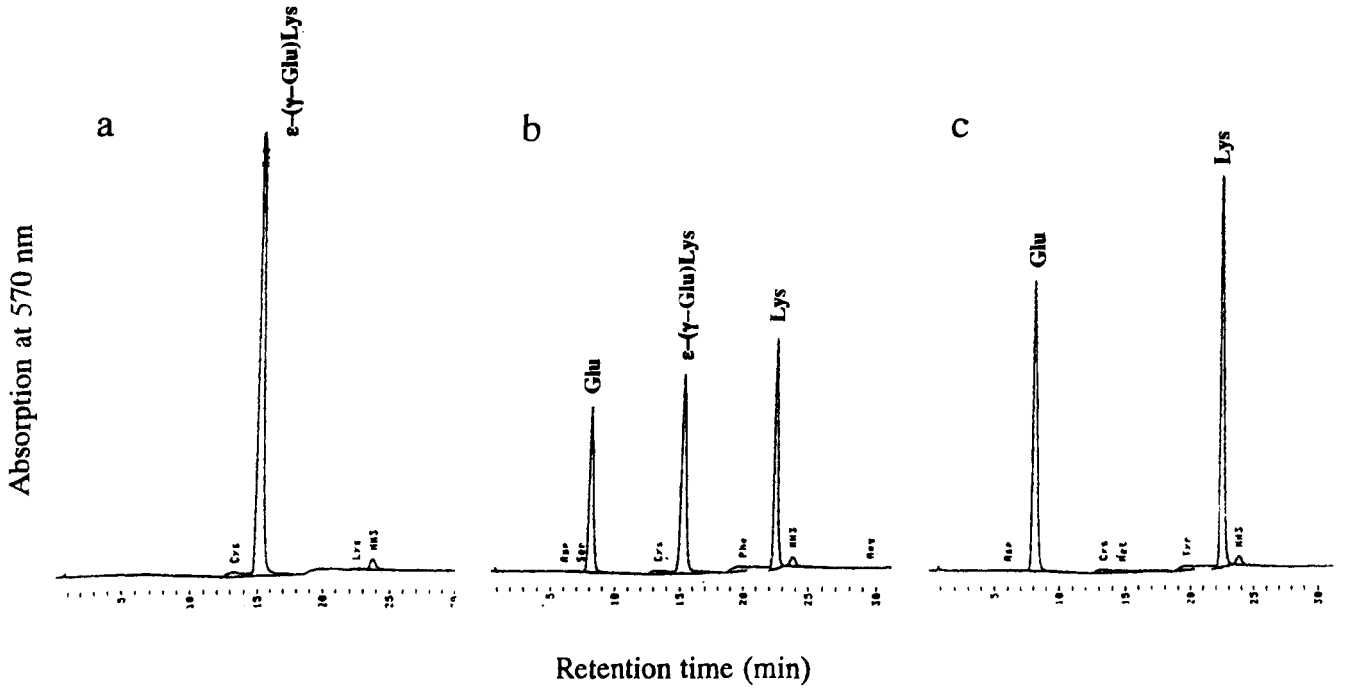

Figure 1. Detection of hydrolytic products of ε-(γ-Glu)Lys dipeptide by γ-GTP. After incubation with bovine γ-GTP (200 μL, 173 units/mL) at 37 °C for certain periods (a, 0 min; b, 1 h; c, 9 h), an aliquot (0.2 mL) of acetic acid was added to 0.23 mM ε-(γ-Glu)Lys solution (1 mL) to stop the enzymatic reaction. Then the reaction mixture was subjected to amino acid analysis.

γ-GTP Digestion of ε-(γ-Glu)Lys Dipeptide. ε-(γ-Glu)-Lys dipeptide (1 mg) was dissolved in 1 mL of distilled water in a microtube, and the pH of the solution was adjusted to pH 7 with 1 N NaOH and/or 1 N HCl. To the solution was added 10 μL of γ-GTP solution (330 units/mL) and mixed vigorously, and the mixture was incubated at 37 °C for certain periods. At certain intervals, an aliquot (100 μL) of the solution was mixed with an equivolume of acetic acid to stop the enzymatic reaction.

Exhaustive Digestion of Cross-Linked Surimi Protein. Exhaustive digestion of TGase-catalyzed cross-linked protein was carried out as previously described (Kumazawa et al., 1993). Cross-linked surimi protein was freeze-dried, and 30 mg of the dried sample was weighed in a plastic test tube. A crystal of thymol and 2 mL of 0.2 N Tris-HCl buffer (pH 8.0) were added into the test tube. Then Pronase was added and the mixture incubated at 37 °C for 24 h. This Pronase addition was repeated once. After inactivation of Pronase by heating at 100 °C for 10 min, leucine aminopeptidase and prolidase were added to the mixture and incubated at 37 °C for 24 h. This was repeated once. After the inactivation of both enzymes, carboxypeptidase A was added to exhaustively digest (for 24 h) the cross-linked protein. After the inactivation of carboxypeptidase A, the mixture was freeze-dried. At the time of determination of ε-(γ-Glu)Lys dipeptide content, the dried powder was dissolved in 7.5 mL of distilled water for HPLC separation.

ε-(γ-Glu)Lys Dipeptide Analysis. Analysis of ε-(γ-Glu)-Lys dipeptide in the samples was performed as described previously with a slight modification (Kumazawa et al., 1993). Briefly, an aliquot (50 μL) of sample was pipetted into a glass test tube, and 450 μL of OPA solution was added and mixed vigorously. Accurately 3 min after the OPA derivatization, 100 μL of the mixture was injected into an HPLC system loaded with a reversed-phase C_{18} column. The OPA-derivatized dipeptide was eluted with a gradient patter from 80% potassium acetate (20 mM, pH 5.5, with 1% THF) against methanol (1% THF) to 100% methanol (1% THF) within 23 min. OPA-derivatized dipeptide was detected at 333 nm for excitation and 450 nm for emission.

Analysis of Amino Acids Liberated. Analysis of amino acids liberated in the γ-GTP reaction was performed with a fully automated amino acid analyzer (L-8500, Hitachi Co.) equipped with a data processor (D-2858, Hitachi). An elution program for common amino acids was used in the determination of amino acids liberated by γ-GTP reaction, and under this condition ε-(γ-Glu)Lys dipeptide coeluted with methionine. Detection was done with ninhydrin derivatization. For the standard amino acids, H-type mixture was used.

RESULTS

Digestion of ε-(γ-Glu)Lys Dipeptides by Bovine γ-GTP. The digestibility of ε-(γ-Glu)Lys dipeptide by bovine γ-GTP was assayed by OPA derivatization with the direct dipeptide analysis using a reversed-phase column, and the liberation of free lysine and glutamic acid was confirmed by amino acid analysis. In the direct ε-(γ-Glu)Lys analysis, the peak corresponding to the dipeptide disappeared (data not shown). In the amino acid analysis, ε-(γ-Glu)Lys dipeptide, eluting at the same retention time as methionine (Met), disappeared on the addition of bovine γ-GTP, and consequently, two new peaks determined as free glutamic acid and lysine in comparison with standard amino acids appeared (Figure 1). Pyroglutamic acid, a known hydrolytic product of ε-(γ-Glu)Lys dipeptide by γ-glutamylamine cyclotransferase, was not detected in the reaction mixture by the analyzer. This indicates that ε-(γ-Glu)Lys dipeptide was definitely cleaved into glutamic acid and lysine by bovine γ-GTP. Enzymatic parameters, K_m and V_{max}, for bovine γ-GTP in the degradation of ε-(γ-Glu)-Lys dipeptide were 4.6×10^{-2} M and 2.0×10^{-6} M/min, respectively.

Digestion of ε-(γ-Glu)Lys Dipeptides by Other γ-GTPs. The digestibility of ε-(γ-Glu)Lys dipeptide by equine, porcine, and *E. coli* γ-GTPs was also assayed by detecting free lysine and glutamic acid on the amino acid analyzer (Table 1). In the case of equine and porcine enzymes, they also reduced the ε-(γ-Glu)Lys peak, together with the appearance of free glutamic acid and lysine. The ε-(γ-Glu)Lys peak did not completely disappear within the same reaction period as bovine γ-GTP, though the same amount of enzymatic activity was added for each origin. The ratios of the cleavage

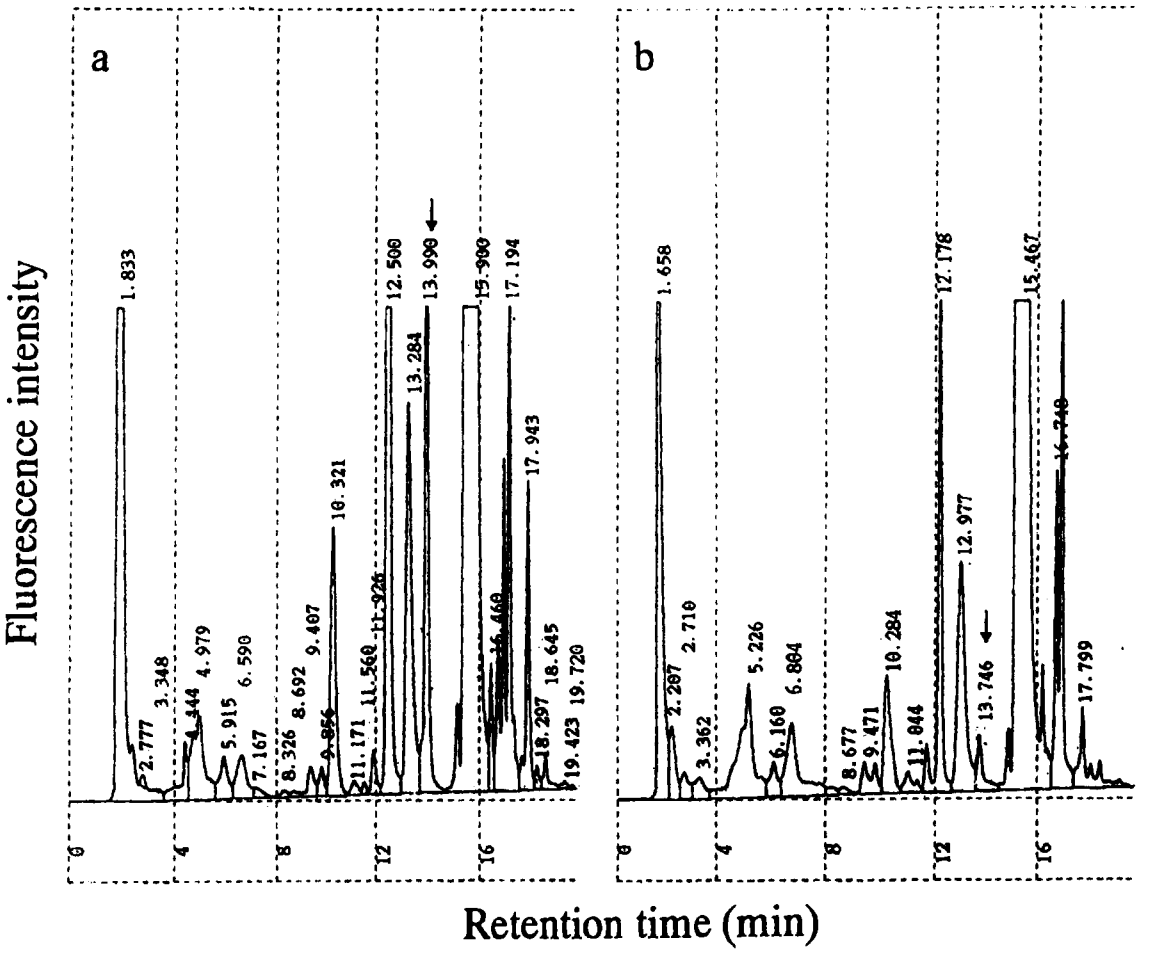

Figure 2. Digestion of ε-(γ-Glu)Lys moiety in hydrolysate of TGase-catalyzed cross-linked surimi protein by γ-GTP. After exhaustive hydrolysis with a series of proteases and peptidases, the sample was incubated at 37 °C for 24 h either in the absence (a) or in the presence (b) of 50 μL of bovine γ-GTP (110 units/mL). Then the reaction mixture was subjected to ε-(γ-Glu)Lys analysis. For details, see Materials and Methods. Arrows indicate the position of the ε-(γ-Glu)Lys dipeptide.

by equine and porcine enzymes to that of the bovine γ-GTP were both about $^1/_{10}$. This difference may reflect the difference in the reactivity of γ-GTP due to different species. Degradation of the dipeptide by *E. coli* γ-GTP was also confirmed by observing the liberation of free glutamic acid and lysine.

Digestion of ε-(γ-Glu)Lys Moiety of Cross-Linked Surimi Protein by γ-GTP. Cross-linked surimi protein was prepared by microbial TGase to produce the ε-(γ-Glu)Lys bonds in the protein, and it was subjected to exhaustive hydrolysis into amino acids by a series of protease and peptidases, such as Pronase, leucine aminopeptidase, carboxypeptidase A, and prolidase. This treatment was performed to resemble gastrointestinal digestion in the body. After the hydrolysis, ε-(γ-Glu)Lys dipeptide uncleavable by common proteases and peptidases in the protein lysate was detected by direct peptide analysis. After incubation with bovine γ-GTP at 37 °C for 12 h, ε-(γ-Glu)Lys dipeptide in the protein lysate diminished almost completely (Figure 2).

DISCUSSION

The bioavailability of ε-(γ-Glu)Lys dipeptide has been proven in experimental animals by several researchers (Mauron, 1970; Waibel and Carpenter, 1972; Raczynski et al., 1975; Finot et al., 1978; Friedmann and Finot, 1990). Administration of isotopic ε-(γ-Glu)[^{14}C]Lys dipeptide in rats also revealed that the isotopic [^{14}C]lysine was accumulated mainly in skin and outer kidney membrane, and thus the dipeptide was proven to be catabolically absorbed in rats. Consequently, a kidney enzyme, γ-glutamylamine cyclotransferase, has been reported to be responsible for the degradation of ε-(γ-Glu)Lys dipeptide (Fink et al., 1980; Iwami and Yasumoto, 1986). On the other hand, it was reported that γ-GTP, whose main catalytic activity is transpeptidation of the γ-glutamyl moiety in glutathione, failed to catalyze the liberation of free lysine from ε-(γ-Glu)Lys dipeptide [personal communication referred to in Folk and Finlayson (1977)]. However, in this study γ-GTP was found to catalyze the cleavage of the ε-(γ-Glu)Lys bond, and the liberation of free lysine and glutamic acid was confirmed (Figure 1). Four γ-GTPs from bovine, equine, and porcine kidney and from *E. coli* cleaved the ε-(γ-Glu)Lys bond, but a difference in the reactivity to

Table 1. Hydrolysis of ε-(γ-Glu)Lys Dipeptide by γ-GTP from Different Origins[a]

γ-GPT origin	act.[b] (units)	ε-(γ-Glu)Lys[c] before	after	Δ[d]	Glu[e] (nmol)	Lys[e] (nmol)
bovine kidney	3.3	2.9	1.1	1.8	1.8	1.7
porcine kidney	2.7	2.6	2.4	0.2	0.17	0.12
equine kidney	3.6	2.6	2.4	0.2	0.22	0.21
E. coli	3.7	3.1	2.7	0.4	0.12	0.14

[a] Reaction conditions: ε-(γ-Glu)Lys dipeptide was incubated with the enzymes at 37 °C for 2.5 h. [b] Activities of γ-GTP added into the reaction mixtures. [c] Amounts of ε-(γ-Glu)Lys before and after γ-GTP treatment in nmol. [d] Difference in ε-(γ-Glu)Lys before and after γ-GTP treatment in nmol. [e] Amounts of Glu and Lys produced after γ-GTP treatment.

ε-(γ-Glu)Lys dipeptide among γ-GTPs was observed (Table 1). Although it was not cited which γ-GTP was used in the previous paper, the difference between the previous and present results may be attributable to the difference in the origins of γ-GTP.

It is reported that the intestinal γ-GTP in rats and chicks was so weak that most ε-(γ-Glu)Lys dipeptide was freed into plasma circulation (Waibel and Carpenter, 1972; Finot et al., 1978). However, there are other reported locations of high γ-GTP activity in the body, and those include the intestinal brush border, pancreatic acinar, ductile epithelial cells, epididymal epithelium, thyroid follicular epithelium, bile duct epithelium, bile canalicular epithelium, choroid plexus epithelium, and many others (Meister et al., 1981). The present study reveals the differences in the reactivity among bovine, porcine, and equine γ-GTPs toward ε-(γ-Glu)-Lys (Table 1). Further, it is known that in lower animals, such as rats and chicks, raw soybean trypsin inhibitors cause detrimental effects; on the other hand, such detrimental effects are not evident in higher animals, such as dogs, calves, pigs, and monkeys (Flavin, 1982; Struthers et al., 1983). The presence of such species differences implies the possibility that in the higher animals the intestinal γ-GTP could effectively cleave ε-(γ-Glu)Lys dipeptide and participate in its digestion in the body. The experiment on γ-GTP digestion after exhaustive hydrolysis of TGase-catalyzed cross-linked surimi protein revealed that the enzyme was fully active and cleaved the ε-(γ-Glu)Lys dipeptide, even in the presence of constituent amino acids (Figure 2). It is thus likely that the TGase-catalyzed cross-linked protein would be digested and lysine in the protein may be possibly utilized in the intestines of higher animals. Together with γ-glutamylamine cyclotransferase and kidney γ-GTP, the cross-linked moiety of ε-(γ-Glu)Lys in TGase-catalyzed cross-linked proteins was almost completely hydrolyzed in the body.

With regard to efficiency in glutamic acid utilization, the γ-GTP digestion seems higher than the γ-glutamylamine cyclotransferase digestion, since an adenosine triphosphate-dependent enzyme, 5-oxoprolinase (EC 3.5.2.9), is required to generate free glutamic acid in the latter case (Meister et al., 1985).

The size of the peptide required to be the substrate of γ-GTP can hardly be predicted at the moment, for only ε-(γ-Glu)Lys dipeptide was tested in this study. Destabilase from medical leeches, on the other hand, was reported to cleave the ε-(γ-Glu)Lys moiety within cross-linked, stabilized fibrin (Baskova and Nikonov, 1985, 1991). Although γ-GTP is categorized as a member of the peptidase family, and it can be easily imagined that γ-GTP would not cleave larger ε-(γ-Glu)-Lys peptides, further study is required to determine how large (or small) peptides should be to become recognized and cleaved as substrates by γ-GTP. Such works are now under way, and the results will be reported soon.

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