

Yapsin 1 immunoreactivity in α -cells of human pancreatic islets: implications for the processing of human proglucagon by mammalian aspartic proteases

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Abstract

Yapsin 1 is an aspartic protease from *Saccharomyces cerevisiae* and belongs to a class of aspartic proteases that demonstrate specificity for basic amino acids. It is capable of processing prohormone substrates at specific basic residue cleavage sites, similar to that of the prohormone convertases, to generate bioactive peptide hormones. An antibody raised against yapsin 1 was previously shown to immunostain endocrine cells of rat pituitary and brain as well as lysates from bovine pituitary secretory granules demonstrating the existence of yapsin 1-like aspartic proteases in mammalian endocrine tissues, potentially involved in peptide hormone production. Here, we show the specific staining of yapsin 1

immunoreactivity in the α -cells of human pancreatic islets. No staining was observed in the β - or δ -cells, indicating a specificity of the staining for glucagon-producing and not insulin- or somatostatin-producing cells. Purified yapsin 1 was also shown to process proglucagon into glucagon *in vitro*, demonstrating that the prototypical enzyme of this subclass of enzymes can correctly process proglucagon to glucagon. These findings suggest the existence of a yapsin 1-like enzyme exclusively in the α -cells of the islets of Langerhans in humans, which may play a role in the production of glucagon in that tissue.

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Introduction

Aspartic proteases represent a class of proteases that use two catalytic triads composed of Asp–Thr–Gly as the functional component of their active site for catalysis. X-ray crystallography and specificity studies revealed that their active sites are generally positioned to accept hydrophobic amino acids; hence, the cleavage specificity of aspartic proteases was thought to be predominant for bulky hydrophobic residues within proteins (Tang 1963), although there are examples of fungal aspartic proteases that can cleave other residues. Coupled with the presence of chymosin (Foltmann 1992) and pepsins (Ryle & Porter 1959) in the stomach to aid digestion and the presence of cathepsin D in the lysosome to aid protein degradation (Erickson & Blobel 1979), it was generally believed that aspartic proteases fell within a group classified as ‘non-specific’ degradation enzymes. However, this is not the case for all aspartic proteases, as evident from the specific processing roles that aspartic proteases play; for example, memapsin 2 (BACE; Vassar *et al.* 1999, Lin *et al.* 2000) and γ -secretase (Wolfe *et al.* 1999) in the pathogenesis of Alzheimer’s disease, or from the role of renin in the

generation of angiotensin I (Fukamizu & Murakami 1995) and its function in the regulation of blood pressure. The yapsins (Cawley & Loh 2011) may also represent an exception to this classification.

The yapsin family of aspartic proteases includes yapsin 1–3 and yapsin 6 and 7 from *Saccharomyces cerevisiae* (*S. cerevisiae*) while other members have been identified in *Candida albicans* (SAP8 and 9; Monod *et al.* 1998), *Saccharomyces pombe* (*Sp Yps1*; Ladds & Davey 2000), *Candida glabrata* (*Cg YPS1*; Dujon *et al.* 2004), and *Aspergillus oryzae* (*opsB*; Kunihiro *et al.* 2002). Yapsin 1 is involved in maintaining cell wall integrity through incorporation and/or retention of glucan (Krysan *et al.* 2005), processing of mucins that activate the MAP kinase signaling pathway (Vadaie *et al.* 2008), and cleavage of GPI-anchored proteins that are shed into the growth medium (Gagnon-Arsenault *et al.* 2008). Unique to the yapsins are their ability to cleave proteins at basic amino acids as opposed to the generally preferred hydrophobic amino acids of other aspartic proteases. Yapsin 1 has been shown to be able to cleave several prohormones including pro-opiomelanocortin (POMC), proinsulin, and ACTH (Cawley *et al.* 1996a) with catalytic efficiencies increasing with increased basic residues

around the scissile bond (Olsen *et al.* 1998). In yeast, yapsin 1 was cloned based on its ability to correctly process pro- α -mating factor at its basic residue cleavage sites (Egel-Mitani *et al.* 1990). As this process is usually performed by Kex2, a subtilisin-like serine protease, it was speculated that yapsins may be backup enzymes for the serine proteases involved in prohormone processing.

Mammalian aspartic proteases with similar properties to the yapsins have been characterized from bovine pituitary intermediate lobe secretory granules (Loh *et al.* 1985) and bovine adrenal chromaffin granules (Azaryan *et al.* 1995). Also, an anglerfish aspartic protease capable of processing prosomatostatin at a basic residue cleavage site has been described (Mackin *et al.* 1991). Indeed, an antibody against yapsin 1 has been used to immunologically identify mammalian yapsin 1-like proteins in bovine and mouse endocrine and neuroendocrine tissue (Cawley *et al.* 1996b). Here, we extend those findings and show yapsin 1-like immunoreactivity exclusively in human pancreatic islet α -cells and that purified yapsin 1 can generate glucagon by processing proglucagon. These results suggest the presence of a yapsin 1-like endoprotease in human pancreatic α -cells with an ability to produce glucagon.

Materials and Methods

Immunohistochemistry

Tissue specimens from six adult human pancreata were obtained from surgical samples removed at operation for pancreatic adenocarcinoma. The study was approved by the local ethics committee at Lisbon University Hospital. The patients had no evidence of any endocrine disease. The specimens were taken from macro- and microscopically normal glandular regions from the body-tail region at least 3 cm distant from the neoplasm. The mean size of the specimens was 1 × 2 cm. The specimens were routinely fixed in 10% buffered neutral formalin for 18–20 h at room temperature and embedded in paraffin. Sections, 5 μ m thick, were cut and attached to positively charged (Superfrost+; Menzel, Braunschweig, Germany) glass slides. Hematoxylin-eosin was used as a routine staining.

Single staining

Single immunofluorescence staining and the indirect two-step peroxidase-labeled dextran-polymer technique (EnVision, DakoCytomation, Glostrup, Denmark; Sabatini *et al.* 1998) with diaminobenzidine as chromogen, using a Dako Autostainer (DakoCytomation), were performed to reveal the distribution pattern of the different endocrine cell types and yapsin 1-immunoreactivity (IR) in the pancreas, as well as to perform the control staining specified below. The sections were pre-treated in a microwave

oven (Philips Whirlpool Nordic AB, Stockholm, Sweden) for 2–5 min at 750 W, using a citrate buffer, pH 6.0, as a retrieval solution.

Double staining

Co-localization studies were performed using immunofluorescence methods with the yapsin 1 antibody and antibodies to various islet hormones, without microwave pretreatment. The yapsin 1 immunofluorescence staining was enhanced by the catalyzed reporter deposition method with biotinyl tyramide. For details of these methods, see Portela-Gomes *et al.* (2000). The secondary antibodies were pre-incubated overnight at 4 °C with non-immune serum, both from the animal species recognized by the other secondary antibody and from the species producing that antibody, at a dilution of 1:10.

The control staining included omission of the primary antibody and replacement of the first layer of antibody by pre-immune or non-immune serum diluted 1:10 or by the diluent alone. To confirm the specificity of the yapsin 1 antibody, absorption tests were carried out by overnight incubation at 4 °C of 5 μ l primary antibody with 45 μ l conditioned culture media containing yapsin 1, before application to the sections. The culture media for the absorption control was obtained from a yeast over-expression system inducible by galactose, as described previously (Azaryan *et al.* 1993, Cawley *et al.* 1995), and demonstrated here to contain yapsin 1 and that it could immuno-absorb the yapsin 1 antibodies. The other primary antibodies were preincubated with the relevant antigen (10 nM/ml diluted antibody solution respectively) before application to the sections. The hormone antigens used were obtained from Sigma Chemical Co.

The rabbit anti-yapsin 1 antibody (MW283; Cawley *et al.* 1995) was used at 1:1600 for the dextran-polymer technique and 1:80 for immunofluorescence. The other primary antibodies were as follows: mouse monoclonal antibodies against human somatostatin (Novo Nordisk S/A, Bagsvaerd, Denmark; clone Som-018; 1:50), guinea pig antibodies against human insulin (P Westermark, Department of Genetics and Pathology, Uppsala, Sweden; code #Ma37; 1:200), and chicken antibodies against human glucagon (1:800) and pancreatic polypeptide (1:100; A Larsson, Department of Medical Sciences, Clinical Chemistry; Uppsala, Sweden). The labeled secondary antisera were biotinylated swine anti-rabbit IgG (DAKO, Glostrup, Denmark), Alexa Fluor 594-labeled streptavidin, Alexa Fluor 488-conjugated goat anti-mouse, anti-guinea pig, and anti-chicken IgG (Molecular Probes, Invitrogen Life Technologies Corporation) and biotinyl tyramide (TSA Biotin System; PerkinElmer, Waltham, MA, USA). For co-localization studies, the sections were examined on a Zeiss META confocal microscope (Carl Zeiss GmbH, Jena, Germany), using argon (488 nm) and HeNe (543 nm) lasers, at a 20× magnification/0.75 numerical aperture (NA) and a 40× magnification/1.3 NA. The pinhole settings were

adjusted to give a depth of image of 0.8 μ m. All images were captured using identical settings at 20 \times magnification in sequential steps, one for each dye. No spectral bleed-through between channels was detected.

DNA constructs and transfection

The cDNA of full-length guinea pig proglucagon in the mammalian expression vector pcDNA 3.1(+) was a gift from Dr S Dhanvantari (Lawson Health Research Institute, Canada). The transfection of the plasmid was accomplished with program T-24 using the Nucleofector 1 device according to the manufacturer (Lonza, Inc., Allendale, NJ, USA).

Cell culture and proglucagon expression

Neuro2a (N2a) cells were transfected with proglucagon or empty vector plasmid and maintained in DMEM containing 10% fetal bovine serum for 24 h. The cells were then washed and incubated with serum-free DMEM (SFM) for 4 h. The conditioned media were collected and concentrated with Amicon Ultracel-3K centrifuge filter units following the protocol of the manufacturer (Millipore, Billerica, MA, USA) and aliquots frozen at -20°C until used.

In vitro processing

Conditioned medium containing proglucagon ($\sim 1.3 \mu\text{M}$) was incubated with and without purified yapsin 1 ($3.7\text{--}6.2 \mu\text{M}$) that had been purified, lyophilized, and stored (Cawley *et al.* 1998), in 50 mM sodium acetate, pH 5.5, supplemented with fresh 1 \times complete protease inhibitor cocktail (G-Biosciences, St. Louis, MO, USA), and 50 μM 4-(2-aminoethyl) benzene-sulfonyl fluoride (AEBSF). The samples were incubated for 18 h at 37°C after which they were stopped by freezing on dry ice until analysis.

Analysis of proglucagon processing

Aliquots of the proglucagon reactions were first analyzed by western blot to demonstrate the decrease in proglucagon substrate and generation of glucagon-sized peptides. For western blot, proteins were separated on 4–12% NuPage acrylamide gels (Invitrogen), transferred to nitrocellulose (0.45 μm), and probed with a rabbit monoclonal antibody against glucagon (Abcam, Inc.) at a dilution of 1:5000. The signal was visualized by the Odyssey infrared scanner (Li-Cor Biosciences, Lincoln, NE, USA) for anti-rabbit secondary antibodies coupled with infrared dyes at 700 or 800 nm, which were used to detect the primary antibodies. As the glucagon antibody cross-reacts with proglucagon, it is expected to cross-react with any glucagon containing peptides including oxyntomodulin and glicentin. However, it does not cross-react with GLP1 or GLP2. Glucagon-IR was also determined in the samples using the glucagon

enzyme immunoassay (EIA; Phoenix Pharmaceuticals, Inc., Burlingame, CA, USA) to determine levels of recovery. The antibody in this kit also cross-reacts with proglucagon and oxyntomodulin (and presumably glicentin) but not with the GLP peptides. Proglucagon samples (40 μl) were further separated by HPLC on a 4.6 \times 250 mm, 5 μm reverse-phase Jupiter C18 column (Phenomenex, Torrance, CA, USA). Buffer A was 0.1% trifluoroacetic acid (TFA) and buffer B was 100% acetonitrile/0.1% TFA and the gradient was 20–60% B in 30 min. Fractions #11–26 were collected, lyophilized, and reconstituted in EIA buffer and assayed by EIA (Phoenix Pharmaceuticals, Inc.) for glucagon-like IR. Under these conditions, oxyntomodulin, a 37 amino acid peptide that contains glucagon (1–29) with an 8 amino acid extension at its C-terminus, and glucagon, as standards (Phoenix Pharmaceuticals, Inc.), eluted in fractions 16 and 19 respectively. Separation of peptides by reverse-phase chromatography depends on hydrophobic interaction;

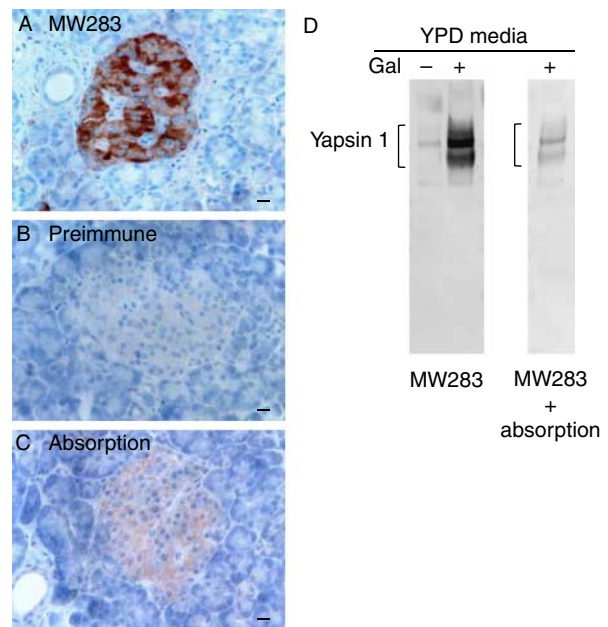


Figure 1 (A–C) Normal human pancreatic islets immunostained with the indirect dextran polymer technique (EnVision) with diaminobenzidine as chromogen using (A) MW283, (B) MW283 preimmune serum, (C) MW283 preincubated with galactose-induced culture medium containing yapsin 1. The slides were counterstained with Mayer's hematoxylin. It is noted that yapsin 1-IR was seen in cells of the islets using the immune serum that were not present when stained with pre-immune serum or where the immune serum was preabsorbed with yapsin 1 protein. (D) Western blot of uninduced (–) and galactose-induced (+) culture media for yapsin 1 stained with the yapsin 1 antibody MW283 alone or absorbed (+ absorption) with galactose-induced culture medium. The reduction in yapsin 1 signal by > 80% in the absorbed antibody sample is found. Bar in A = 50 μm ; bar in B and C = 40 μm . Full colour version of this figure available via <http://dx.doi.org/10.1530/JOE-11-0121>.



Figure 2 Confocal microscopic images of normal human pancreatic islet double immunostained for yapsin 1 (Alexa Fluor 594, red) and glucagon (Alexa Fluor 488, green). All glucagon α -cells show yellow to yellow–green color indicating co-localization of glucagon and yapsin 1-IR in human islets. Bar = 40 μ m. Full colour version of this figure available via <http://dx.doi.org/10.1530/JOE-11-0121>.

hence, elution of glicentin, a 69 amino acid glucagon-containing peptide, in fraction 19, where the 29 amino acid glucagon peptide elutes, is highly improbable and therefore not tested.

Results

Immunohistochemistry

Distinct yapsin 1-immunoreactive cells were seen in the human pancreatic islets. The immunoreactivity was strong and diffusely distributed throughout the cytoplasm, involving cell processes when present. The cells belonging to the exocrine parenchyma did not show any immunoreactivity (Fig. 1A–C). Yapsin 1-IR was found in virtually all glucagon-immunoreactive cells and vice versa (Fig. 2). The intracytoplasmic distributions of the yapsin 1-IR and glucagon immunoreactivities were in agreement, but the yapsin 1-IR often appeared weaker than glucagon. Glucagon-expressing α -cells have a varying localization pattern in human pancreatic islets; they may have a peripheral distribution in the islets, but they may also occur in the middle of the islets mainly adjacent to the perivascular stroma. Yapsin 1-IR was not present in any of the insulin-, pancreatic polypeptide- (not shown), or somatostatin-immunoreactive cells (Fig. 3). In double immunofluorescence staining, our control tests showed that omission of one of the primary antibodies gave a staining pattern corresponding to that of the remaining primary antibody. No immunoreactivity was observed in islets stained with the yapsin 1 preimmune serum. The yapsin 1 antibody, preincubated with galactose-induced medium containing yapsin 1, gave rise to only weak immunoreactive cells, demonstrating the specific reduction of the yapsin 1-IR signal by the pre-absorbed antiserum (Fig. 1D).

Production and processing of proglucagon

The presence of yapsin 1-IR in glucagon-expressing cells prompted us to test whether yapsin 1 could process proglucagon *in vitro*. Proglucagon was found in the culture media of N2a cells transfected with proglucagon cDNA (Fig. 4A). Whereas the N2a cells in general gave non-specific

bands in the high molecular mass range, only the cells transfected with proglucagon-pcDNA3.1 showed a strong staining of glucagon-IR at the expected size of \sim 22 kDa for proglucagon (Fig. 4A). Incubation of the medium containing proglucagon with purified yapsin 1 caused the disappearance of proglucagon and the generation of smaller glucagon-IR products, some consistent with the size of glucagon (Fig. 4B). In this case, the amount of glucagon-IR in the reaction with and without yapsin 1 treatment was 32.1 and 28.3 ng/ml, respectively, which was equivalent to the amount in the starting material. Analysis of the peptides generated by yapsin 1 by HPLC followed by EIA identified a peak in fraction 19 that is identical to the elution profile of glucagon standard (Fig. 5). No peak was observed in fraction 16, indicating that the glucagon-IR products did not contain oxyntomodulin. Of the 1.28 ng of glucagon-IR injected, \sim 0.24 ng was recovered in fraction 19, representing a \sim 20% recovery of glucagon from the digestion of proglucagon by yapsin 1.

Discussion

The cloning of yapsin 1, originally referred to as *yap3* (Egel-Mitani *et al.* 1990, Bourbonnais *et al.* 1993) as it was the third aspartic protease to be cloned from *S. cerevisiae*, provided evidence of a new subclass of aspartic proteases in that it was able to process a yeast prohormone, pro- α -mating factor, correctly at paired-basic residue cleavage sites. It did so in the absence of Kex2, a prohormone processing enzyme of the serine protease class, the first protease to be unequivocally identified both biochemically and genetically, as a prohormone processing enzyme (Julius *et al.* 1984, Thomas *et al.* 1988). The fact that yapsin 1 could perform this function also introduced the idea, at that time, that aspartic proteases may have more specific roles in endocrinology. Indeed, a mammalian aspartic protease was identified and characterized several years prior to the cloning of yapsin 1 (Loh *et al.* 1985).



Figure 3 Confocal microscopic images of normal human pancreatic islet double immunostained for yapsin 1 (Alexa Fluor 594, red) and insulin (Alexa Fluor 488, green; left panel) or somatostatin (Alexa Fluor 488, green; right panel). Insulin or somatostatin-positive cells (green) do not co-stain with yapsin 1-IR (red) in human islets. Bar = 30 μ m. Full colour version of this figure available via <http://dx.doi.org/10.1530/JOE-11-0121>.

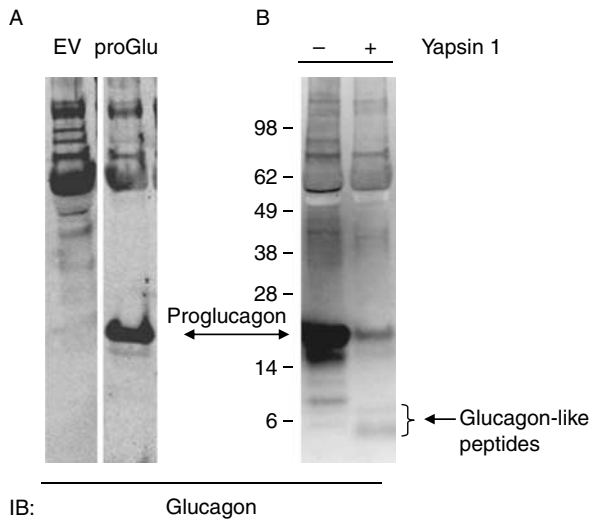


Figure 4 *In vitro* processing of proglucagon by purified yapsin 1. (A) Western blot for proglucagon in conditioned media collected from Neuro2a cells transfected with EV (left) or proglucagon-pcDNA3.1 (right). (B) The medium was incubated with (+) or without (-) purified yapsin 1 enzyme and the products were analyzed by western blot. Analysis shows the disappearance of the proglucagon substrate and production of glucagon-sized immunoreactive bands (+) produced specifically in the yapsin 1 containing sample.

That enzyme, now termed yapsin A, to indicate it as the first mammalian yapsin-like enzyme, was shown to process prohormones such as POMC (Loh *et al.* 1985), provasopressin (Parish *et al.* 1986, Loh *et al.* 1988), and proinsulin (Loh *et al.* 1985). In addition, its unique high molecular mass relative to other aspartic proteases of ~68–70 kDa is similar to the mass for deglycosylated yapsin 1 (~65 kDa; Cawley *et al.* 1995). In almost all aspects, yapsin A appears to be a homolog of yapsin 1, even immunologically. An antibody was generated against yapsin 1 and used to analyze mammalian tissue by western blot and immunohistochemistry (Cawley *et al.* 1996b). Staining of yapsin 1-IR was seen in cells from anterior and intermediate lobes of the pituitary in addition to selected cells in areas of the brain such as mouse arcuate nucleus and hippocampus and the rat supraoptic and paraventricular nuclei, cortex, striatum, and reticular nucleus as well as in extracts from bovine anterior pituitary secretory granules. However, while specific staining was observed in these tissues, characterization of yapsin A at the nucleotide level has not been achieved; hence, the amino acid sequence of yapsin A is still unknown.

In this study, we analyzed human islets with the yapsin 1 antiserum. Our results show strong, specific staining of yapsin 1-IR in glucagon-producing cells only. The specific nature of the staining, in that it was absent from β - and δ -cells, indicated that possibly a yapsin A enzyme was present in human islet α -cells where glucagon is produced and secreted. We tested whether yapsin 1, as the prototypical enzyme for this subclass of enzymes, could generate glucagon from

proglucagon, because in many cases tested, yapsin 1 could process prohormone substrates in a similar pattern to that of yapsin A. We used conditioned medium containing proglucagon and indeed, when incubated with purified yapsin 1, glucagon was produced. It is important to note that while glucagon was identified as the final product, it is likely that yapsin 1 cleaved the proglucagon on the carboxyl side of the paired-basic residue cleavage site and that carboxypeptidase E-like activity from the N2a conditioned media likely removed the extended basic residues to generate authentic glucagon. This processing pattern would be consistent with yapsin 1 specificity because both the N- and the C-termini are flanked by Lys-Arg cleavage sites that are readily recognized by yapsin 1 (Cawley *et al.* 1996a). Furthermore, an additional Arg present in the P2' position at the C-terminal cleavage site favorably enhances the processing by yapsin 1 as has been demonstrated previously (Ledgerwood *et al.* 1996, Olsen *et al.* 1998). The selectivity of the enzyme is also observed as two Arg residues (Arg⁶⁹-Arg⁷⁰) found within the glucagon sequence (NCBI accession # P55095) were not cut.

These results raise the question as to whether a yapsin-like enzyme contributes to the important physiological function of glucagon production *in vivo*. One can speculate that similar to the ability of yapsin 1 to process pro- α -mating factor in the absence of Kex2, mammalian yapsin A may be able to participate in the production of glucagon in α -cells in times where PC2, the mammalian homolog of Kex2 involved in glucagon production *in vivo* (Rouille *et al.* 1997), is downregulated. Efforts at cloning this enzyme are underway.

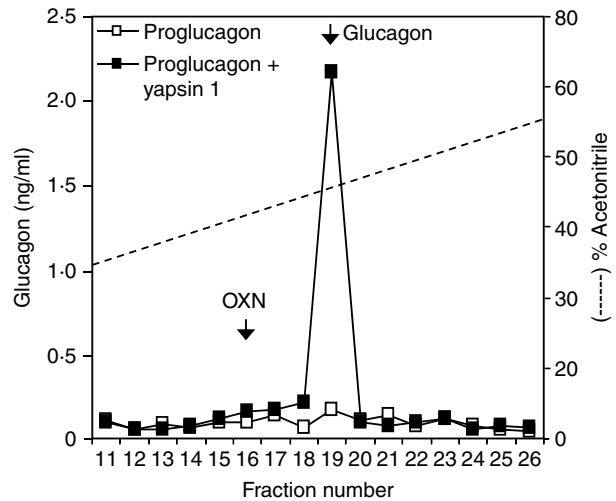


Figure 5 HPLC analysis of glucagon generated by yapsin 1. Aliquots from the *in vitro* processing reactions were subjected to HPLC to separate the peptides on a reverse-phase C18 column. Enzyme-linked immunoassay of the fractions identified that authentic glucagon was recovered in fraction 19 only from the sample incubated with yapsin 1. The arrows indicate where glucagon and oxyntomodulin standards elute (Phoenix Pharmaceuticals, Inc.).

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

N X C and H L performed and analyzed the *in vitro* processing of proglucagon. G P G performed the immunohistochemistry. Y P L supported and advised throughout the project and edited the manuscript. N X C wrote the manuscript.

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