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Purified yeast aspartic protease 3 cleaves anglerfish pro-somatostatin I and II at di- and monobasic sites to generate somatostatin-14 and -28

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Anglerfish somatostatin-14 (SS-14) and somatostatin-28 (aSS-28) are derived from pro-somatostatin I (aPSS-I) and pro-somatostatin II (PSS-II), respectively. Purified yeast aspartic protease 3 (YAP3), was shown to cleave aPSS-I at the Arg^{81}-Lys^{82} to yield SS-14 and Lys^{-1}SS-14. In contrast, YAP3 cleaved aPSS-II only at the monobasic residue, Arg^{73} to yield aSS-28. Since the paired basic and monobasic sites are present in both precursors, the results indicate that the structure and conformation of these substrates dictate where cleavage occurs. Furthermore, the data show that YAP3 has specificity for both monobasic and paired basic residues.

Pro-somatostatin; Yeast; Aspartic protease; Prohormone processing; Somatostatin

1. INTRODUCTION

Neuropeptides and peptide hormones are synthesized as larger precursors which are cleaved most commonly at paired basic residues and in some cases at monobasic sites to yield the biologically active peptides [1,2]. Such a mechanism of biosynthesis of various bioactive peptides extends across different phyla, including yeast, mollusks, lower vertebrates and mammals [1,3,4]. Specific enzymes that recognize dibasic and monobasic residues are involved in these processing events [2–4,5]. Among the first to be identified and cloned was a yeast subtilisin-related serine protease, Kex2, which cleaves the paired basic residues of pro-α-mating factor to generate α-mating factor [5,6]. Subsequently two mammalian homologues of Kex2, PC1/PC3 and PC2 have been cloned and shown to process a number of prohormones [4,7–12]. Recently, a yeast aspartic protease encoded by YAP3, a non-essential gene, has been identified in Kex2-deficient mutant and shown to process pro-α-mating factor [13]. YAP3 has been overexpressed, purified and characterized as a ~70,000 mol.wt. glycoprotein and is enzymatically active at a pH optimum of 4.0–4.5. It has been shown to cleave the paired basic residues of the mammalian adrenocorticotropin/endorphin prohormone, pro-opiomelanocortin, in vitro [14]. From gene deletion experiments, YAP3 has been implicated to also cleave at a monobasic site of anglerfish pro-somatostatin II to yield the mature hormone, somatostatin-28, when this prohormone gene was transfected into yeast cells [15]. However, confirmation that this cleavage activity is mediated by YAP3 awaits in vitro studies with the purified enzyme. In the present study, we show that purified YAP3 cleaves anglerfish pro-somatostatin I at the appropriate pair of basic residues to yield somatostatin-14, and pro-somatostatin II at the monobasic residue to yield somatostatin-28, in vitro.

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2. MATERIALS AND METHODS

2.1. *Preparation of [^{35}S]Cys labeled anglerfish pro-somatostatin I (aPSS-I) and II (aPSS-II)*

Anglerfish islet prohormones, aPSS-I and aPSS-II were radiolabeled by incubating islet tissue with [^{35}S]Cys. Tissue homogenates were subjected to gel filtration and the prohormones were isolated by high-performance liquid chromatography (HPLC) as previously described [16,17]. Samples containing aPSS-I and aPSS-II were partially dried down using a speed vac concentrator, and used for incubation with YAP3 enzyme. The specific activity of each of the precursors aPSS-I and aPSS-II was estimated using protein concentrations determined from integration of the A_{210} UV absorbance peak for each precursor, obtained from HPLC chromatograms during purification. The estimated specific activity of aPSS-I and aPSS-II used in the experiments presented was 7.61×10^5 cpm/μg protein, and 2.92×10^5 cpm/μg protein, respectively.

2.2. *Purification of yeast aspartic protease 3 (YAP3) activity*

Yeast (*S. cerevisiae*), strain BJ3501, was transformed with the YAP3 gene under the control of the galactose promoter as described previously [14] and induced by galactose for ~20 h. A significant amount of expressed YAP3 was secreted into the growth medium (Cawley, N.X., unpublished data) and was used as the source of enzyme. The

growth medium was collected after removing the cells by centrifugation at 2,000 × *g*. Freshly prepared inhibitors were added (1 mM phenylmethanesulfonyl fluoride, 1 mM iodoacetamide and 1 μg/ml aprotinin final concentration) to the growth media. YAP3 enzymatic activity from the growth medium was purified by concanavalin A (ConA, Pharmacia LKB Biotechnology AB, Uppsala, Sweden) affinity chromatography as described previously [14]. The ConA column eluate was desalted on a PD-10 (Sephadex G-25M, Pharmacia LKB) column with 20 mM ammonium bicarbonate, 0.02% Tween and concentrated on a Centricon C-30 membrane filter (Amicon Division, Beverly, MA 01915). YAP3 activity was determined by the rapid I^{125}-β_h-lipotropin assay as described in Azaryan et al. [14]. I^{125} β_h-LPH cleaving enzymatic activity was found in the ConA purified medium of induced yeast, which was completely inhibited by pepstatin A. No proteolytic activity was found in the growth medium of uninduced yeast.

2.3. *Incubation of aPSS-I and aPSS-II with YAP3 and analysis of products formed*

Purified YAP3 (~100 ng) was incubated with ~4,500 cpm [^{35}S]Cys labeled aPSS-I or aPSS-II for 1, 2, and 6 h at 37°C in 0.1 M Na citrate buffer, pH 4.0 with and without 10^{-4} M pepstatin A (Sigma, St. Louis, MO). The reaction was terminated by the addition of acetic acid to a final concentration of 3 M. The products formed were analyzed by reverse phase HPLC as previously described [18]. A 0.1% trifluoroacetic acid/acetonitrile gradient system was used for HPLC and 0.8 ml fractions were collected for scintillation counting.

3. RESULTS

Incubation of aPSS-II with YAP3 enzyme resulted in the cleavage of this substrate to yield somatostatin-28 (aSS-28), but not [Tyr^7,Gly^{10}]SS-14 (Fig. 1). The lack of formation of [Tyr^7,Gly^{10}]SS-14 was further confirmed using a shallower HPLC gradient (identical to Fig. 2) which better separated [Tyr^7,Gly^{10}]SS-14 from aSS-28 (data not shown). Pepstatin A completely inhibited the generation of aSS-28 from aPSS-II, indicating that the enzymatic activity was due to the aspartic protease, YAP3 (Fig. 1). No aSS-28 was formed when aPSS-II

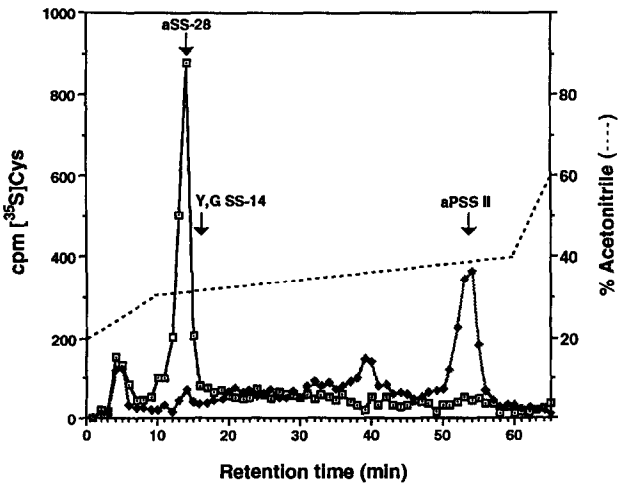

Fig. 1. HPLC profiles of products generated by incubating aPSS-II with YAP3 for 6 h at 37°C in pH 4.0 buffer without (□) and with (■) pepstatin A. The arrows show the standards and the dotted line the acetonitrile gradient which ran from 20 to 29% in 10 min, 29 to 33% in 20 min, and 33 to 39% in 30 min. Y = Tyr; G = Gly.

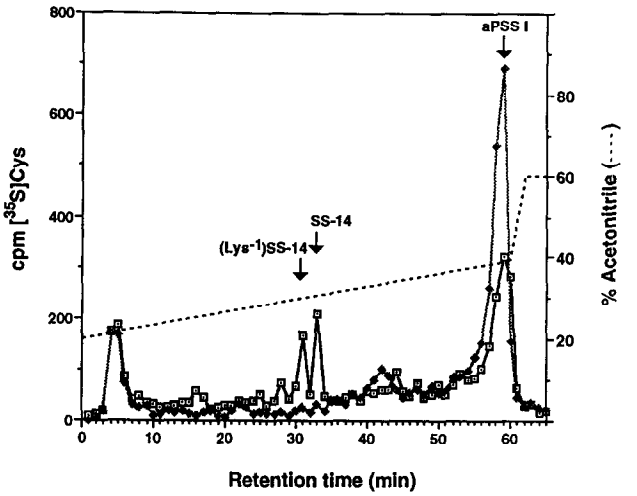

Fig. 2. HPLC profiles of products generated by incubating aPSS-I with YAP3 for 6 h at 37°C in pH 4.0 buffer without (□) and with (■) pepstatin A. The arrows show the standards and the dotted line the acetonitrile gradient which ran from 20 to 39% in 60 min.

was incubated without YAP3. These results indicate that YAP3 cleaved aPSS-II at the monobasic site, Arg^{73}, to yield aSS-28, but not at the dibasic, Arg^{86}-Lys^{87} site to yield [Tyr^7,Gly^{10}]SS-14.

Fig. 2 shows the cleavage of aPSS-I by YAP3 at the paired residues Arg^{81}-Lys^{82}, to yield SS-14 and [Lys^{-1}] SS-14. The formation of these two products indicates that YAP3 cleaved on the carboxyl side and in between the Arg^{81}-Lys^{82} pair of aPSS-I. However, the aSS-28 product was not observed after a 1 h, 2 h or 6 h incubation of PSS-I with YAP3. Pepstatin A completely inhibited the formation of SS-14 confirming that the cleavage at the paired basic residues of aPSS-I was due to the aspartic protease YAP3.

The time course of generation of SS-14 and aSS-28 from aPSS-I and aPSS-II, respectively, by YAP3, was analyzed (Fig. 3). Production of SS-14 and aSS-28 was linear between 1 and 6 h under the incubation conditions used ($r^2 = 0.98$, slope = 54.1 for SS-14 and $r^2 = 0.99$, slope = 271.6 for aSS-28). The rate of generation of aSS-28 from aPSS-II by YAP3 was five-fold greater than the rate of SS-14 formation from aPSS-I. Given that the specific activity of aPSS-II is 2.6-fold lower than aPSS-I, this is in fact an underestimation.

4. DISCUSSION

Previous studies have identified a subtilisin-related PC2-like serine protease, purified from anglerfish islet secretory granules, as the endogenous enzyme that cleaves aPSS-I to yield SS-14 [19]. This enzyme also cleaved aPSS-II to yield [Tyr^7,Gly^{10}]SS-14, a tetradecapeptide analogue of SS-14 [19]. A different enzyme was purified from anglerfish islet secretory granules that processes aPSS-I to SS-28 at the monobasic residue.

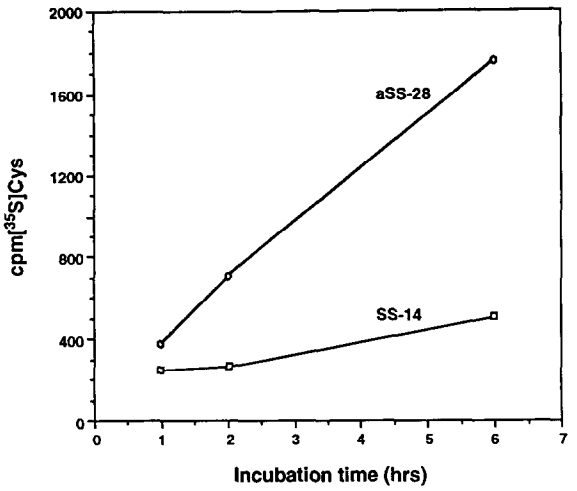

Fig. 3. Time course of generation of aSS-14 from aPSS-I (□) and aSS-28 from aPSS-II (○).

This enzyme was characterized as a 39,000 mol.wt. aspartic protease with a pH optimum of 4.2 [20]. More recently, by the use of deletion mutants of the YAP3 gene in yeast, it was suggested that an aspartic protease encoded by this gene was involved in the processing of aPSS-II to aSS-28 when the prohormone gene was transfected into yeast cells [15]. Results from the present in vitro study unequivocally demonstrate that purified YAP3 can specifically cleave the monobasic Arg^{73} residue of aPSS-II to generate aSS-28. YAP3 did not appear to cleave the monobasic site of aPSS-I, since even after only a 1 h incubation the aPSS-I form of SS-28 was not observed, although it is possible that the SS-28 if generated, was rapidly converted to SS-14. Conversely, YAP3 cleaved the dibasic residues Arg^{81}-Lys^{82} of aPSS-I, but not the equivalent dibasic pair of aPSS-II to generate [Tyr^{7},Gly^{10}]SS-14 despite the fact that the amino acid sequences of the two prohormones are identical in the three amino acids upstream and six amino acids downstream from the basic pair cleavage site. These data clearly indicate that the structure and conformation of the substrates play a very important role in dictating specificity of cleavage of prohormones. The presence of a single basic residue in the −6 position (relative to aSS-28) in aPSS-II is consistent with the rules governing monobasic cleavage sites [21] and accounts for the processing at Arg^{73}. The lack of cleavage at the monobasic site of aPSS-I may be due to the presence of His at position −6 instead of a basic residue. Site-directed mutagenesis studies involving substitution of amino acids adjacent to the cleavage sites of human prosomatostatin have identified a region located between Asn^{-12} and Gly^{+2} that is important for processing the prohormone to SS-14 and SS-28. Specifically, mutation of the nucleotides encoding prolines (proline is a known β-turn blocker) for Ala at the −5 and −9 positions within the PSS gene, resulted in a dramatic decrease in processing when the mutated gene was transfected into Neuro 2A cells, supporting a role for secondary and tertiary structure in directing cleavage of the prohormone [22]. It is perhaps the differences in the amino acids further upstream from the dibasic cleavage sites that account for the differences in cleavage of the dibasic residues of the two prohormones by YAP3.

The ability of YAP3 to cleave aPSS-II at the monobasic site five times more rapidly than at the dibasic site of aPSS-I raises the possibility that the anglerfish SS-28 generating aspartic protease purified from islets may be a fish homologue of YAP3. Indeed, a mammalian aspartic protease, pro-opiomelanocortin converting enzyme (EC 3.4.23.17), purified from bovine intermediate pituitary, with very similar specificity for paired basic residues of pro-opiomelanocortin, pH optimum and size to YAP3, and shares immunological cross reactivity with an antibody against YAP3 has been identified [14,23]. Work is now in progress to determine if pro-opiomelanocortin converting enzyme will also cleave at the monobasic residue of aPSS-II. While YAP3 may cleave the monobasic residue of aPSS-II more rapidly than the dibasic residues of aPSS-I, this relative difference may be only for these two substrates. Further work is necessary to determine if YAP3 is generally more efficient in cleaving at monobasic than dibasic residues of various prohormones.

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