Purification and Characterization of the Yeast Glycosylphosphatidylinositol-anchored, Monobasic-specific Aspartyl Protease Yapsin 2 (Mkc7p)*

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The Saccharomyces cerevisiae YPS2 (formerly MKC7) gene product is a glycosylphosphatidylinositol-linked aspartyl protease that functions as a yeast secreter. Here, the glycosylphosphatidylinositol-linked form of yapsin 2 (Mkc7p) was purified to homogeneity from the membrane fraction of an overexpressing yeast strain. Purified yapsin 2 migrated diffusely in SDS-polyacrylamide gel electrophoresis (molecular mass ~ 200 kDa), suggesting extensive, heterogeneous glycosylation. Studies using internally quenched fluorogenic peptide substrates revealed cleavage by the enzyme carboxyl to Lys or Arg. No cleavage was seen when both Lys and Arg were absent. A significant enhancement was seen with multiple basic residues. No cleavage was seen when both Lys and Arg were absent. No cleavage was seen by the enzyme carboxyl to Lys or Arg. No cleavage was seen by the enzyme carboxyl to Lys or Arg. No cleavage was seen by the enzyme carboxyl to Lys or Arg. No cleavage was seen by the enzyme carboxyl to Lys or Arg.

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2 The abbreviations used are: GPI, glycosylphosphatidylinositol; βAPP, β amyloid precursor protein; IQ substrates, internally quenched fluorogenic peptide substrates; EDANS, 5-[(2-aminoethyl)amino]naphtthalene-1-sulfonyl group; DABCYL, 4-[(diethylamino)phenyl]-azo-benzoyl group; TPCK, N-tosyl-l-phenylalanine chloromethyl ketone; TLCK, N-tosyl-l-lysine chloromethyl ketone; PAGE, polyacrylamide gel electrophoresis; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; Tricine, N-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)glycine; Mes, 4-morpholineethanesulfonic acid.

3 Residues surrounding the cleavage site are designated P_1^-P_2^-P_3^-P_4^-P_5^-P_6^-P_7^-P_8^-P_9^- with the scissile bond between residues P_1 and P_2 of x. 
Expression plasmid was called pG-MKC7. Buffer A containing 1% Triton X-100, incubated for 30 min on ice, and corresponding fragment from the original $Bgl$ structural gene under control of the $YPS2$ promoter. This final expression plasmid was called pG-MKC7.

**Assay of Yapsin 2 Proteolytic Activity for the Purification**—Yapsin 2 proteolytic activity was assayed as described (5). Reaction mixtures (20 μl) contained 100 mM sodium citrate (pH 4.0), 5 mM CaCl₂, 0.01% (v/v) Triton X-100, and 20 μg IQ substrate 2: Arg-Glu-EDANS)-Ser-Leu-Asp-Lys-Arg-Glu-Ala-Glu-Ala-Lys(DABCYL)-Arg. After enzyme addition, reaction mixtures were incubated at 37 °C for 30 min and terminated by adding 680 μl of 1 M Tris base (pH 10). Fluorescence was determined using a Perkin-Elmer LS-5B fluorimeter ($\lambda_{ex}$ 338 nm; $\lambda_{em}$ 490 nm). 1 unit of activity was defined as an increase in fluorescence per min equivalent to that of 1 pmol of Glu(EDANS).

**Purification Procedure**—Strain HKY26 containing plasmid pG-MKC7 was grown to a density of 6 × 10⁷ cells/ml in 3 liters of synthetic complete medium with uracil. Cells were harvested and washed by centrifugation at 3,000 × g. 15 ml of packed cells were resuspended with 24 ml of Buffer A (50 mM HEPES, pH 7.5, 10 mM EDTA, 0.5 mM phenylmethlysulfonyl fluoride, 0.1 mM TPC, 0.1 mM TLCK, 1 mM benzamidine hydrochloride), mixed with 15 ml of glass beads (0.5 mm), and broken by vortexing 60 × 20 s at 4 °C (all subsequent steps were carried out at 4 °C). Broken cells were centrifuged for 3 min at 500 × g to remove cell debris (clear lysate). The supernatant fraction was centrifuged for 1 h at 100,000 × g, and the resulting pellet was washed with 50 ml of Buffer A containing 0.5 M NaCl by recentrifugation at 100,000 × g for 30 min. The final pellet (crude membrane fraction) was solubilized in 50 ml of Buffer A containing 1% Triton X-100, incubated for 30 min on ice, and centrifuged at 100,000 × g for 1 h. The pellet was back-extracted with 30 ml of Buffer A and the two supernatant fractions were pooled (solubilized membrane fraction) and used for further purification.

The solubilized membrane fraction was applied to a 40-ml (3 × 5 cm) Fast Flow Q-Sepharose column equilibrated with Buffer B (50 mM HEPES, pH 7.0, 0.5 mM EDTA, 10 mM NaCl, 0.5% Triton X-100). The column was washed with 2 column volumes of Buffer B and 8 column volumes of Buffer B2 (Buffer B1 minus EDTA) and eluted with 200 ml of Buffer C (Buffer B2 plus 0.2 M NaCl). 2 μl of each fraction (10 ml) were diluted 10-fold and assayed, and fractions containing activity were pooled. Each pool was adjusted to a final pH of 7.5 and centrifuged at 100,000 × g for 30 min. 3 μl of each fraction containing one-half of 50 ml HEPES (pH 9.0) containing 0.5 M NaCl and applied to a Cu⁺²-chelating Sepharose column (1.8 × 6 cm) equilibrated with Buffer D (50 mM HEPES, pH 7.5, 0.1% Triton X-100, 0.3 mM NaCl). 4 ml of Buffer D and 5 ml of Buffer E (50 mM HEPES, pH 7.5, 0.7% CHAPS, 0.3% NaCl), and eluted with 50 ml of Buffer F (50 mM sodium acetate, pH 5.5, 0.7% CHAPS, 0.3% NaCl). 2 μl of each fraction (2.5 ml) were diluted 10-fold and assayed. Fractions containing activity were pooled (~30 ml) and concentrated with a Centriprep-100 cartridge (Amicon, Inc.) to a final volume of ~400 μl. The concentrated sample was split in half, and each half was applied to a Sephacryl S-300 (1 × 49 cm) gel filtration column equilibrated with Buffer E. 2 μl of each fraction (80 μl) were diluted 10-fold and assayed. Fractions containing activity were pooled and concentrated using a Centriprep-100 cartridge to produce the final fraction. Protein was measured using the BCA assay (Pierce). SDS-PAGE was carried out by the method of Laemmli (27).

**Determination of Cleavage Sites in IQ Substrates—**0.04 μg/ml yapsin 2 and 200 ~ 400 pmol of IQ substrate were mixed and incubated in standard reaction buffer for 60 min. The reaction was stopped by addition of 0.1 N NaOH and subjected to automated Edman degradation by the Protein and Carbohydrate Core Facility at the University of Michigan.

**Determination of V_{max}K_{m} for IQ Substrates—**V_{max}K_{m} was determined using pseudo-first order kinetics. At substrate concentrations much lower than $K_{m}$, production formation follows a simple first order curve (28). Reactions (600 μl) were started by addition of yapsin 2 (final concentration, 0.02 to 1 μg/ml). 100-μl samples taken at times between 0 and 40 min were quenched with 600 μl of 1 M Tris-HCl (pH 10). Raw fluorescence data were fitted to an exponential curve with floating end points using Kaleidagraph version 3.0. Substrate concentrations were between 1 and 2 μM. All progress curves had r > 0.99 (Pearson’s r value).

**Results**

**Purification of Yapsin 2**—Yapsin 2 was purified from an overexpressing strain lacking the YPS1 gene (see “Experimental Procedures”). The major proteolytic activity (70% of total activity) was recovered in the membrane fraction (100,000 × g pellet) and solubilized by 1% Triton X-100 (5). Detrimental extraction resulted in approximately 10-fold purification relative to the cleared lysate (data not shown). Further purification of yapsin 2 from the solubilized membrane fraction is described in detail under “Experimental Procedures.” Although pepstatin A affinity column chromatography has been used to purify several aspartyl proteases, including yapsin 1 (30, 31), less than 10% of yapsin 2 activity bound to the column and the yield of enzyme activity upon elution was extremely low. Therefore, we purified yapsin 2 using conventional column chromatography. Following anion exchange separation on Q-Sepharose, step elution with Cu⁺²-chelating Sepharose resulted in a major diffuse band migrating at ~100 kDa that was visualized by SDS-PAGE (Fig. 1A, lane 9) and coincided with the peak of activity (data not shown). Note that although equal amounts of activity (10⁶ units) were loaded onto a 4-μl sample loaded in lanes 2 (detergent-solubilized membranes), 3 (Q-Sepharose pool), and 4 (chelating Sepharose 6B peak) in Fig. 1A, the heterogeneity and diffuse migration of the yapsin 2 protein prevented the band from being obvious until elution from Q-Sepharose. The chelating Sepharose 6B peak was subjected to Sephacryl S-300 gel filtration (Fig. 1A, lane 5), and the peak of yapsin 2 activity coincided with the appearance of this diffuse band which was separated from other, lower molecular weight polypeptides that eluted later (Fig. 2, A and B). The peak fractions were pooled to avoid the lower molecular weight polypeptides and the pooled enzyme was subjected to both
Specificity of Yeast GPI-linked Aspartyl Protease Yapsin 2

TABLE I

Purification of yapsin 2 from Triton X-100 solubilized membrane fraction

| Step | Protein Volume | Activity | Specific activity | Yield |
|------|----------------|----------|------------------|-------|
| Triton X-100- solubilized membrane fraction | 100 | 120 | 8.7 | 0.87 | 100 |
| Q-Sepharose | 15 | 63 | 6.3 | 4.3 | 72 |
| Cu2+-charged chelating | 1.5 | 35 | 1.9 | 13 | 22 |
| Sepharose | ND | 0.68 | 1.7 | ND | 20 |
| Centriprep-100 (concentration) | 0.57 | 8.2 | 1.1 | 19 | 13 |
| Centriprep-100 (concentration) | 0.45 | 0.68 | 0.86 | 19 | 9.9 |

*ND, not determined.*
Specificity of Yeast GPI-linked Aspartyl Protease Yapsin 2

Fig. 3. pH dependence of yapsin 2 activity. A, pH dependence of yapsin 2 cleavage of IQ substrate 2 (A) and IQ substrate 21 (B). Assay mixtures contained 20% sucrose, 10 mM CaCl$_2$, 0.007% CHAPS, 50 mM yapsin 2 cleavage of IQ substrate 2 (A) and IQ substrate 21 (B). Activity was normalized to 1.

Concentrations of pepstatin A at pH 6.0. Therefore, efficient cleavage of substrate 2 was inhibited by high hydrophilic residues in substrate sequences (Fig. 4, see below). Leupeptin (Fig. 4), TLCK, TPCK, phenylmethylsulfonyl fluoride, and E64 (data not shown) did not inhibit yapsin 2 activity.

Addition of EDTA, EGTA, Cu$^{2+}$, Mg$^{2+}$, Ca$^{2+}$, or Mn$^{2+}$ had no effect on the activity (data not shown), despite the fact that yapsin 2 bound to Cu$^{2+}$-charged chelating Sepharose.

Substrate Specificity—The natural substrates of yapsin 2 and yapsin 1 are still unknown. However, overexpression of either YPS1 or YPS2 suppressed the sterility of a kex2 null mutant (5, 6), suggesting that these enzymes can cleave pro-$\alpha$ factor at the Kex2 cleavage sites. In addition, combined deletion of the YPS1 and YPS2 genes blocked cleavage of human $\beta$APP at the $\alpha$-secretase site, suggesting that human $\beta$APP is a substrate of yapsin 2 (12, 15). Therefore, the substrate specificity of yapsin 2 was tested using a series of IQ substrates based on the pro-$\alpha$ factor sequences (set A in Table II) and human $\beta$APP sequences (set B in Table II). Cleavage sites were determined by automated Edman degradation (Table III, “Experimental Procedures”).

Set A tested the ability of yapsin 2 to cleave sequences containing 1, 2, or 3 basic amino acid residues. Yapsin 2 cleaved all sequences in set A, except for the one containing a Pro-Arg factor sequence, with similar $V_{\text{max}}/K_m$ values, corresponding to a $K_{\text{cat}}/K_m$ of $-10^7$ s$^{-1}$ M$^{-1}$, assuming a molecular weight of 64,000 and that all yapsin 2 molecules were active. In every case where cleavage was observed, the cleavage site was carboxyl to the most COOH-terminal basic residue, Arg, Lys, or ornithine, revealing that yapsin 2 prefers a basic residue at P$_1$ but fails to discriminate between structurally dissimilar basic residues at P$_1$ (Table III, substrates 3, 5, 6, 8, 10, 11, and 13). In addition, these results demonstrate that yapsin 2 cleavage was essentially no selective at P$_2$ with the exception of excluding Pro at P$_2$. The further observation that cleavage was always seen carboxyl to the most COOH-terminal basic residue in sequences with multiple basic residues suggests strongly that basic residues are excluded from P$_1$ by yapsin 2 (Table III, substrates 3, 5, 6, 8, and 10).

In the case of set B (Table II), six IQ substrates based on human $\beta$APP cleavage sites were tested. Significant cleavage was observed only in substrates 15, 17, and 21, which contained a Lys residue, consistent with a requirement by yapsin 2 for a basic residue at P$_1$. Cleavage in each case tested occurred carboxyl to the Lys residue (Table III, substrates 15 and 21). At pH 4.0, $V_{\text{max}}/K_m$ for these substrates was 2 orders of magnitude less than for the substrates based on pro-$\alpha$ factor-based sequences, although as pointed out previously (see Fig. 3), $V_{\text{max}}/K_m$ for substrate 21 increased by $-20$-fold at pH 6.0. The lower cleavage efficiency for set B versus set A substrates was probably due to the effect of sequence context rather than
of yapsin 2 with N-glycanase, which removes Asn-linked oligosaccharides (34), resulted in an increased mobility in SDS-PAGE, but not to the predicted molecular weight. Furthermore, the band was still diffuse (data not shown), suggesting that yapsin 2 also contains heterogeneous Ser/Thr-linked oligosaccharides. Yapsin 2 eluting late in Sephacryl S-300 gel filtration (Fig. 2B) exhibited higher mobility in SDS-PAGE than early eluting enzyme, also consistent with extensive, heterogeneous glycosylation. A high degree of heterogeneous glycosylation is characteristic of yeast periplasmic and cell wall proteins and is a feature of yapsin 1 as well (8, 35).

Yapsin 2 exhibits a bell-shaped pH dependence of $V_{\text{max}}/K_m$ and is sensitive to pepstatin A as found for other aspartyl proteases (29, 32, 33). The bell-shaped pH profile of aspartyl proteases has been shown to depend on the $pK_a$ values of two conserved catalytic aspartyl residues; one of these is deprotonated with $pK_a$ between 1 and 3, and the other protonated with a higher $pK_a$ of approximately 4.5 (29, 32). The $pK_a$ values measured for cleavage by yapsin 2 of IQ substrate 2, whose sequence is based on one of two Kex2p cleavage sites in pro-$\alpha$ factor, were 3.8 and 4.3, resulting in a very narrow pH optimum of approximately 4.0 (Fig. 3A). Because care was taken to measure the pH dependence under conditions in which the enzyme was stable, the pH profile most likely reflects the intrinsic pH dependence of the reaction between free enzyme and substrate as opposed to an effect of pH on folding. It is possible that the high value of the lower $pK_a$ observed with IQ substrate 2 may reflect titration of an acidic side chain in the substrate itself. Indeed, the quite different pH dependence of cleavage of IQ substrate 21 (Fig. 3B), presumably due to titration of His residues in the substrate, demonstrates the existence of dramatic substrate-dependent effects on catalysis by yapsin 2. Human immunodeficiency virus-I protease has been shown to exhibit different pH dependence of $k_{\text{cat}}$ and $K_m$ for substrates that differ by a single acidic side chain at $P_2^\prime$, although the shift in pH optimum for $k_{\text{cat}}/K_m$ was not nearly as large (29). The shift of the pH optimum of yapsin 2 to near neutrality with substrate 21, which represents the $\alpha$-secretase cleavage site in human $\beta$APP, is likely a relevant factor in the efficiency of cleavage of human $\beta$APP by yapsin 2 in yeast in vivo (12). Furthermore, the ability of yapsin 2 to cleave certain substrates effectively in a higher pH range may be relevant to the physiological function of yapsin 2 as a processing enzyme in the secretory pathway or at the cell surface in yeast. Further study will be needed to determine how substrate sequence and pH affect substrate binding and catalysis by yapsin 2.

Summarizing the analysis of our specificity studies, yapsin 2 appears to require a basic residue at $P_1$. This requirement seems to be based on the charge rather than on the structure of the side chain, because structurally dissimilar basic side chains gave comparable $V_{\text{max}}/K_m$ values. Yapsin 2 does not appear to cleave between basic residues, however, suggesting that the enzyme excludes basic residues at $P_1$. This distinguishes yapsin 2 from yapsin 1, which has been shown to cleave between basic residues in certain substrates (9). However, analysis of strains lacking $YPS1$, $YPS2$, or both genes indicates that yapsin 2 and yapsin 1 are at least partially physiologically redundant in yeast and therefore must cleave at least an overlapping pool of substrates (7). In addition, 25 residues predicted on the basis of a structural model of yapsin 1 to interact with substrate side chains from $P_2^\prime$ to $P_6$ (11), 20 are conserved in yapsin 2 (5). Direct comparison of the two enzymes using identical substrates should lead to a better understanding of the relative specificities of the two enzymes.

The distinction between the specificity of yapsin 2 and that of the serine protease Kex2p is more extensive. First, Kex2p is

### Table II

**Specificity of Yeast GPI-linked Aspartyl Protease Yapsin 2**

| Substrate No. | Sequence | $V_{\text{max}}/K_m$ $\min ^{-1}$ |
|---------------|----------|-----------------------------------|
| IQ substrate: set A | | |
| 1 | RBPYMRRKAEAJR | 2.7 ± 0.1 |
| 2 | RBSLDRKAEAJR | 2.7 ± 0.1 |
| 3 | RIBDPYRKEAEABR | 5.1 ± 0.1 |
| 4 | RIBDRKKEAEABR | 3.0 ± 0.3 |
| 5 | RIBRKKEAEABR | 2.7 ± 0.2 |
| 6 | RIBYRKEAEABR | 3.6 ± 0.3 |
| 7 | RIBBDYKEAEABR | 2.9 ± 0.1 |
| 8 | RIBRDYKEAEABR | 5.2 ± 0.2 |
| 9 | RIBBDYKEAEABR | 3.1 ± 0.1 |
| 10 | RIBRDYKEAEABR | 4.5 ± 0.3 |
| 11 | RIBDRYKEAEABR | 8.1 ± 0.7 |
| 12 | RIBRYEAEABR | 2.4 ± 0.2 |
| 13 | RIBRYEAEABR | 4.5 ± 0.2 |
| 14 | RIBRYEAEABR | No detectable cleavage |
| IQ substrate: set B | | |
| 15 | AcGBEVEKMDEAEEFGJ | 0.030 ± 0.003 |
| 16 | AcGBEVIDMEAEEFGJ | No detectable cleavage |
| 17 | AcGBEVEKMDDEAEEFGJ | 0.051 ± 0.003 |
| 18 | AcGBEVIDMDEAEEFGJ | No detectable cleavage |
| 19 | AcGBVIAVTVIJIJ | No detectable cleavage |
| 20 | AcBBVHHQKLVFJR | 0.001 ± 0.006 |
| 21 | | |

to a preference for Arg over Lys at $P_1$, because yapsin 2 cleaved Lys-Arg (substrates 1, 2, 3, and 5), Arg-Arg (substrate 8), Lys-Lys (substrate 6), Arg-Lys (substrate 10), and Lys-Orn (substrate 7) sites in set A with comparable efficiencies (Table II). Yapsin 2 was capable of cleaving at the human $\beta$APP $\alpha$-secretase cleavage site, consistent with the observed cleavage of $\beta$APP when expressed in yeast (12–15). Yapsin 2 did not cleave the $\alpha$-secretase substrate, substrate 15, correctly (i.e. carboxyl to the Met residue) and did not cleave the $\gamma$-secretase substrate, substrate 20, at all (Table II).
highly specific for Arg at P₁. Substitution of Lys for Arg at P₁ in
otherwise identical substrates not only reduces $k_{cat}/K_m$ by
~500–3600-fold, it also results in a change in the rate-limiting
step of the reaction from deacylation to acylation (36). Whereas
Kex2p exhibits large decreases in $k_{cat}/K_m$ when residues other
than Lys or Arg are present at P₂, yapsin 2 appears to be
largely insensitive to the nature of the P₂ residue, with the
exception of Pro (see below). For example, on IQ substrates 3
and 13, Kex2p exhibited $k_{cat}/K_m$ values of 2.5 × 10⁷ and 5.6 ×
10⁴ M⁻¹ s⁻¹, respectively, a 450-fold difference (23). In contrast,
yapsin 2 exhibited essentially identical $V_{max}/K_m$ values (and
therefore $k_{cat}/K_m$ values) for the two substrates (Table II).
Conversely, Kex2p is relatively permissive for Pro at P₂, which
reduces $k_{cat}/K_m$ by only ~7-fold (24), whereas in the case of
yapsin 2, $V_{max}/K_m$ was reduced by >10⁵-fold by substitution of
Pro at P₂.

The differences between yapsin 2 and Kex2p are not surpris-
ing in the sense that the enzymes represent distinct mechanis-
tical and evolutionary classes of proteases. However, the fact that
both yapsin 2 and yapsin 1 can suppress phenotypic defects of
kex2 null mutant strains and that null mutations of the YPS1
and YPS2 genes result in more severe phenotypes when com-
bined with kex2 null mutations suggests that the three
ezymes cleave at least some common substrates in vivo (5).
However, whereas Kex2p can be accurately categorized by its
specificity for pairs of basic residues, the data presented here
argue that yapsin 2 is a monobasic specific enzyme. What could
be responsible for the ability of yapsin 2 to cleave Kex2 sub-
strates accurately? The exclusion of basic residues from the P₁
position in yapsin 2 plausibly accounts for the ability of the
zymes cleave at least some common substrates

| IQ No. | substrate sequence | 1 | 2 | 3 | 4 | 5 | 6 |
|-------|-------------------|---|---|---|---|---|---|
| 3     | RβYKR | EAEABR | R (25) | — | β (100) | Y (12) | K (25) | R (12) |
| 5     | RβKKR | EAEABR | E (51) | A (66) | E (41) | A (44) | — | — |
| 6     | RβYKK | EAEABR | R (284) | — | β (105) | Y (49) | E (44) | R (156) |
| 8     | RβYRR | EAEABR | E (65) | A (73) | E (64) | A (73) | — | — |
| 9     | RβYRK | EAEABR | R (151) | — | β (162) | Y (90) | K (87) | K (73) |
| 10    | RβYRK | EAEABR | E (82) | A (113) | E (66) | A (93) | — | R (69) |
| 11    | RβYRK | EAEABR | R (253) | — | β (137) | Y (89) | R (177) | R (232) |
| 13    | RβYPR | EAEABR | E (90) | A (110) | E (78) | A (95) | — | — |
| 15    | AcGBEVK | DMAEFJG | M (104) | D (78) | A (98) | E (50) | F (25) | — |
| 21    | AcRBVHIQQ | LVFJR | L (84) | V (101) | F (64) | R (124) | — | — |

**TABLE III**

Cleavage sites of yapsin 2 in IQ substrates

Arrows indicate the predominant cleavage site. Exhaustive reactions of substrates with yapsin 2 and NH₂-terminal sequence analysis of products
were carried out as described under "Experimental Procedures."—unidentified peak, most likely corresponding to either PTH-Glu(EDANS) or
PTH-Lys(DABCYL). Data for substrate 21 are from Ref. 12 and are included for comparison. Non-standard amino acid abbreviations are as in
the legend to Table II.

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Specificity of Yeast GPI-linked Aspartyl Protease Yapsin 2

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