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New Proteolytic Enzymes in Yeast

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Yeast mutants lacking three proteolytic enzymes—proteinase B, carboxypeptidase Y, and carboxypeptidase S—have been constructed. Search for new proteolytic activities in these mutants with the aid of chromogenic peptide substrates developed for serum proteinases led to the detection of new proteolytic activities, active in the neutral pH range. Sephadex chromatography of a 100,000*g* supernate of mutant extracts, tests against four different substrates, and partial characterization of their sensitivity to various inhibitors indicate multiple activities. Two activities, called proteinase M and proteinase P, were found in the sedimentable membranous fraction of mutant extracts.

The fundamental role proteolytic enzymes play in cellular regulation has been recognized in recent years. In the yeast *Saccharomyces cerevisiae* seven proteolytic enzymes have been found—two endoproteinases, two carboxypeptidases, three aminopeptidases, and one dipeptidase [for reviews see (1, 2)]. From *in vitro* studies proteinase B, an enzyme located in the vacuole and the only known endoproteinase active at neutral pH, was considered to be involved in several specific intracellular regulatory processes such as activation of chitin synthetase, a vital process during yeast budding (3) and the inactivation of a variety of enzymes unnecessary under certain growth conditions [for reviews see (1, 2)]. However, *in vivo* experiments using proteinase B mutants made involvement of the proteinase B in these specific processes unlikely [for reviews see (1, 2)]. Therefore we searched for new proteolytic enzymes active at neutral pH which might be responsible for proteolytic processes outside the vacuole. Chromogenic peptide substrates designed for the relatively specific serum proteinases were applied during this search. As a prerequisite for these studies, a triple mutant devoid of proteinase B, carboxypeptidase Y, and carboxypeptidase S was constructed, ensuring lack of activity of known enzymes in the cell against the new substrates used.

MATERIALS AND METHODS

Chemicals. Bz-Pro-Phe-Arg-Nan[1] (Chromozym PK), Tos-Gly-Pro-Lys-Nan (Chromozym PL), Cbz-Val-Gly-Arg-Nan (Chromozym TRY), and Tos-Gly-Pro-Arg-Nan as well as carboxypeptidase Y were obtained from Boehringer (Mannheim, FRG). Bz-Phe-Val-Arg-Nan (S-2160), Bz-Ile-Glu-Gly-Arg-Nan (S-2222), H-D-Phe-Pip-Arg-Nan (S-2238), H-D-Val-Leu-Lys-Nan (S-2251), and H-D-Val-Leu-Arg-Nan (S-2266) were purchased from Kabi (München, FRG). Suc-Ala-Ala-Ala-Nan was from Protein Research Foundation (Osaka, Japan). Bz-Asp-Trp-Arg-Nan was from Vega (Tucson, Ariz.). Bz-Tyr-Nan and $PhCH_2SO_2F$ were from Serva (Heidelberg, FRG). Cbz-Gly-Gly-Leu-Nan, Suc-Ala-Ala-Pro-Phe-Nan, and Suc-Gly-Gly-Phe-Nan were from Bachem (Bubendorf, Switzerland). Azocoll was obtained from Calbiochem (Giessen, FRG). Chymostatin and pepstatin were a generous gift from Professor Dr. H. Umezawa. Morpholinopropane sulfonic acid and microsomal leucine aminopeptidase were from Sigma (Taufkirchen, FRG). Sephadex G-150 was from Pharmacia (Freiburg, FRG). Purified proteinase B was a generous gift from Dr. E. Kominami. All yeast growth

[1] Abbreviations used: Nan, 4-nitroanilide; Suc, succinyl; Bz, benzoyl; Tos, tosyl; Cbz, benzyloxycarbonyl; Pip, pipecolyl; $PhCH_2SO_2F$, phenylmethylsulfonylfluoride; buffer A, 0.1 M morpholinopropane sulfonic acid/KOH buffer, pH 7.2; buffer B, 0.1 M morpholinopropane sulfonic acid/KOH buffer, pH 7.5.

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media were from Difco (Roth, Karlsruhe, FRG). All other chemicals, which were of highest purity available, were obtained from Merck (Darmstadt, FRG).

Yeast strains and growth conditions. Triple mutants lacking the three proteolytic enzymes proteinase B, carboxypeptidase Y, and carboxypeptidase S, due to lesions in their structural genes (D. H. Wolf and C. Ehmann, in preparation), were constructed by crossing strain HP 232-2B (*α, prb 1*) (4, 5) with strain cps 14-9A (*a, prc 1, cps 1*) (6), (D. H. Wolf and C. Ehmann, in preparation). Diploids were allowed to sporulate and haploids carrying the *prb 1, prc 1,* and *cps 1* mutations were selected. Strain BYS 232-14-23 (*α, prb 1, prc 1, cps 1*) was used in these studies.

YPD medium contained 1% yeast extract, 2% peptone, and 2% glucose. YPE medium contained 2% ethanol (v/v) instead of glucose. When grown in YPD medium cells were harvested in early stationary phase (20 to 22 h of growth at 30°C). When grown in YPE medium cells were harvested in early logarithmic phase (20 to 25 h of growth at 30°C).

Preparation of soluble extract and sedimentable membranous fraction. Cells were harvested by centrifugation, washed twice with distilled water, and resuspended in 0.1 M morpholinopropane sulfonic acid/KOH buffer, pH 7.2 (buffer A), using a ratio of 1 g of cells/ml buffer. When soluble cell extract was used in the experiments, YPD-grown cells were broken by passing them twice through a French pressure cell (Aminco, Silver Spring, Md.) with 20,000 lb/in^2. The extract was centrifuged for 30 min at 100,000*g* and at 4°C in a 50 Ti rotor. The clear supernatant extract was collected. When the membrane fraction was used in the experiments, YPE-grown cells were broken and centrifuged as described above. The pellet fraction was washed three times with buffer A by centrifugation at 100,000*g*. The washed membranous fraction from 1 g of cells was resuspended in 0.3 ml buffer A.

Gel filtration. Gel filtration using Sephadex G-150 was done in a 1 × 100-cm column. One milliliter of cell extract was applied to the column. Flow rate was 5.6 ml per hour. Buffer was buffer A.

Enzyme assays. Proteolytic activities against the chromogenic peptide substrates in the soluble and membranous fraction of cell extracts were measured at 25°C by adding the enzyme sample (up to 0.05 ml) to 0.5 ml of 1 mM peptide solution in 0.1 M morpholinopropane sulfonic acid/KOH buffer, pH 7.5 (buffer B). Cbz-Gly-Gly-Leu-Nan was solubilized in 15% dimethylformamide (v/v) in buffer, Suc-Ala-Ala-Pro-Phe-Nan and Suc-Gly-Gly-Phe-Nan were solubilized in 5% dimethylformamide (v/v) in buffer. When measuring soluble enzyme activity, change in absorbancy at 405 nm caused by release of 4-nitroaniline was followed kinetically in an Eppendorf spectrophotometer (Hamburg, FRG). In cases where turbidity occurred during the assay, absorbancy of the mixtures was measured after a certain test time (between 1 and 4 h, depending on the activity) after centrifugation of the precipitating material. Proteolytic activity in the membranous fraction, visible as liberated 4-nitroaniline at 405 nm, was determined after shaking the incubation mixture for 3 h. Using crude extract linearity of the tests has been established for high protein concentrations. This holds true for both time dependence and protein added to the test. Specific activity is expressed as nanomoles 4-nitroaniline liberated per minute per milligram protein assuming a molar absorption coefficient of 9500 liters × mol^{-1} × cm^{-1} for 4-nitroaniline at 405 nm based on the data of Erlanger *et al.* (7). When enzyme activities against the various substrates were followed after column chromatography, because of costs, the test volume was reduced and conditions were slightly changed. The following were incubated: 0.1 ml of 1 mM peptide solution, 0.06 ml of column eluate, and 0.04 ml of buffer A; 0.2 ml of buffer B was added before activity determination. The presence of 4-nitroanilides of contaminating peptides with free α-L-amino termini susceptible to aminopeptidase activity was checked with microsomal leucine aminopeptidase. When necessary, endoproteolytic impurities in the commercial aminopeptidase preparations were inhibited by chymostatin and phenylmethylsulfonylfluoride. For testing 4-nitroanilides with leucine aminopeptidase, conditions used were as described above. Proteinase B activity was determined as described by Saheki and Holzer (8) using Azocoll as substrate. Carboxypeptidase Y was measured according to Aibara *et al.* (9) using Bz-Tyr-Nan as substrate. Carboxypeptidase S activity was determined according to Wolf and Weiser (10) using Cbz-Gly-Leu as substrate.

Inhibition of proteolytic activity was measured after 30 min of preincubation at 25°C with the respective inhibitors. Reaction was started by addition of substrate.

Genetic procedures. Genetic procedures were followed as outlined in Ref. (11).

Protein determination. Protein was estimated according to the method of Lowry *et al.* (12) using crystalline bovine serum albumin as standard.

RESULTS

The 100,000*g* supernatant extract of the triple mutant BYS 232-14-23 (*α, prb 1, prc 1, cps 1*) grown into stationary phase was tested against a variety of chromogenic proteinase substrates designed for plasma proteinases and other endoproteolytic enzymes. As can be seen from Table I, proteolytic activity can be measured against all substrates except H-D-Phe-Pip-Arg-

TABLE I

PROTEOLYTIC ACTIVITIES IN 100,000*g* SUPERNATE OF AN EXTRACT OF STRAIN BYS 232-14-23 (*α, prb 1, prc 1, cps 1*)

Peptide substrate	sp act[a]	Percentage activity remaining after addition of			
		$PhCH_2SO_2F$	Chymostatin	EDTA	$HgCl_2$
Bz-Pro-Phe-Arg-Nan	0.69	88	88	17	76
Tos-Gly-Pro-Lys-Nan	0.1	100	100	25	100
Tos-Gly-Pro-Arg-Nan	0.1	66	100	17	100
Bz-Ile-Glu-Gly-Arg-Nan	0.81	100	73	66	96
Bz-Phe-Val-Arg-Nan	0.33	83	83	24	100
H-D-Phe-Pip-Arg-Nan	<0.1	—	—	—	—
H-D-Val-Leu-Lys-Nan	0.25	80	80	40	100
H-D-Val-Leu-Arg-Nan	0.32	100	83	66	66
Cbz-Val-Gly-Arg-Nan	0.19	100	87	63	100
Bz-Asp-Trp-Arg-Nan	0.12	—	27	83	—
Suc-Ala-Ala-Ala-Nan	<0.1	—	—	—	—
Cbz-Gly-Gly-Leu-Nan	0.37	88	22	97	47
Suc-Ala-Ala-Pro-Phe-Nan	0.23	65	66	28	108
Suc-Gly-Gly-Phe-Nan	0.12	—	—	—	—

Note. Cells were grown into stationary phase. Tests were done with 2 to 4 mg of protein as outlined under Materials and Methods. Inhibitor concentrations were: $PhCH_2SO_2F$, saturated by addition of crystals; chymostatin, 0.25 mg/ml; EDTA, 2 mM; $HgCl_2$, 0.2 mM. Activity without inhibitors is set 100%.

[a] Specific activity given as (nmol × min^{-1} × mg^{-1}).

Nan and Suc-Ala-Ala-Ala-Nan. Activity is highest against Bz-Ile-Glu-Gly-Arg-Nan and Bz-Pro-Phe-Arg-Nan.

There was no significant change in any of the activities measured, when extracts of cells growing logarithmically on glucose as carbon source were tested (data not shown). Incubation of cell extracts with sodium dodecyl sulfate (0.5%, 3 h), a powerful method of activating proteinase B (4, 13), did not lead to any increase in activity against the proteinase substrates measured. In contrast, activity against a variety of 4-nitroanilide substrates dropped significantly (data not shown).

No clear correlation between the activity measured with a certain peptide substrate and a single enzyme or a group of enzymes can be seen in the crude extract after adding inhibitors specific for the functional groups of known proteinases (Table I). Among these were $PhCH_2SO_2F$ which abolishes the activity of serine proteinases, Hg^{2+} ions which inhibit SH-proteinases, the chelating agent EDTA, which is able to inhibit metalloproteinases (14, 15), and chymostatin as a potent inhibitor of papain, chymotrypsin, and cathepsin B-like proteinases (16). From the inhibition studies using these inhibitors, it seemed likely that more than one enzyme in crude extracts of the triple mutant is able to cleave the chromogenic substrates. Therefore, we tried to separate the possible activities on Sephadex G-150.

Tests of the column fractions from gel filtration were done with the two chromogenic peptide substrates of high cleavage rate harboring a basic amino acid at the cleavage site, namely Bz-Pro-Phe-Arg-Nan and Bz-Ile-Glu-Gly-Arg-Nan. In addition, we also used Cbz-Gly-Gly-Leu-Nan and Suc-Ala-Ala-Pro-Phe-Nan thereby introducing a neutral and an aromatic amino acid at the cleavage site. The distribution of proteolytic activity after chromatography measured with these four substrates can be seen in Figs. 1A-D as well as Figs. 2A-D, left axis. These figures also show the remaining proteolytic activity after addition of the proteinase inhibitors EDTA, $PhCH_2SO_2F$, and chymosta-

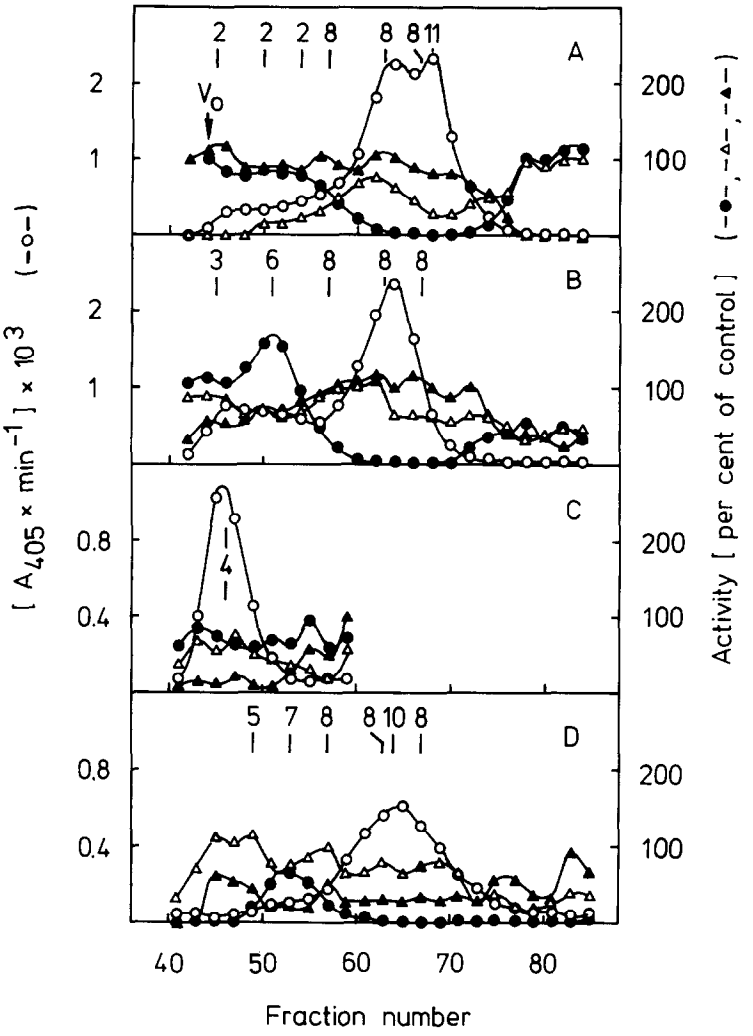

FIG. 1. Gel filtration of a 100,000*g* supernate of a cell extract of strain BYS 232-14-23 (*α, prb 1, prc 1, cps 1*). Sephadex G-150 chromatography was done as outlined under Materials and Methods. The substrates were added after 30 min of preincubation of the proteolytic activities with or without the inhibitors. Inhibitor concentrations were: $PhCH_2SO_2F$, saturated by addition of crystals; chymostatin, 0.25 mg/ml; EDTA, 1 mM. Proteinase substrates were: (A) Bz-Pro-Phe-Arg-Nan; (B) Bz-Ile-Glu-Gly-Arg-Nan; (C) Cbz-Gly-Gly-Leu-Nan; (D) Suc-Ala-Ala-Pro-Phe-Nan. In (A)-(C) activity was measured after 4 h, in (D) after 24 h. Left axis: ○, Proteolytic activity (control). Right axis: ●, EDTA; △, $PhCH_2SO_2F$; ▲, chymostatin. (Data are shown as percentage of control activity). The arrow marks the exclusion volume (V_0). Numbers of possible activities correspond to numbers in Table II.

tin (Figs. 1A-D, right axis). In preliminary experiments Co^{2+} ions were found to have a partly inhibitory and partly activating effect on the new proteolytic activities. Therefore, we measured activity with the four substrates also after incubation of the column fractions in the presence of 1 mM $CoCl_2$ (Figs. 2A-D, right axis).

Two main proteinase activity peaks can be shown with the three substrates Bz-Pro-Phe-Arg-Nan (A), Bz-Ile-Glu-Gly-Arg-Nan (B), and Suc-Ala-Ala-Pro-Phe-

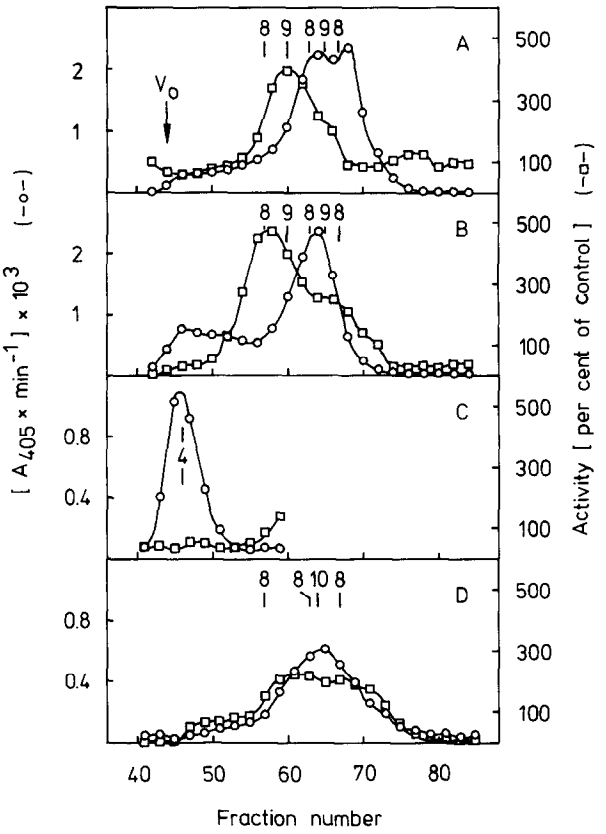

FIG. 2. Gel filtration of a 100,000*g* supernate of a cell extract of strain BYS 232-14-23 (*α, prb 1, prc 1, cps 1*). For experimental conditions see legend to Fig. 1. Concentration of $CoCl_2$ was 1 mM. Proteinase substrates were: (A) Bz-Pro-Phe-Arg-Nan; (B) Bz-Ile-Glu-Gly-Arg-Nan; (C) Cbz-Gly-Gly-Leu-Nan; (D) Suc-Ala-Ala-Pro-Phe-Nan. In (A)-(C) activity was measured after 4 h, in (D) after 24 h. Left axis: O, Proteolytic activity (control). Right axis: □, $CoCl_2$ (data are shown as percentage of control activity). The arrow marks the exclusion volume (V_0). Numbers of possible activities correspond to numbers in Table II.

Nan (D). One activity appears at or near the exclusion volume of the Sephadex column exhibiting a molecular weight higher than 500,000 and the second activity is eluted in the range between molecular weights 40,000 and 150,000. Only one proteinase activity peak near the void volume of the column is visible when substrate Cbz-Gly-Gly-Leu-Nan (C) is used. At first we called the high-molecular-weight activity proteinase D and the activity of lower-molecular-weight proteinase E (1, 2). However, the visible peaks seem to describe overall proteolytic activities which may be subdivided into several possible activities when the proteinase inhibitors EDTA, $PhCH_2SO_2F$, and chymostatin as well as Co^{2+} ions are used to achieve further differentiation. The multiple proteolytic activities subsequently found were marked in (Figs. 1A-D, 2A-D). They are summarized in Table II together with their approximate molecular weights and their inhibition patterns. The molecular weights were determined in a calibration run under identical conditions using Dextran blue, catalase, aldolase, hemoglobin, ovalbumin, chymotrypsinogen A, and cytochrome *c* as molecular-weight markers (data not shown). According to these two criteria, different molecular weight and different inhibition pattern, 11 activities

TABLE II

PROTEOLYTIC ACTIVITY PATTERN IDENTIFIED IN A 100,000*g* SUPERNATE OF THE STRAIN BYS 232-14-23 (*α, prb 1, prc 1, cps 1*) WITH VARIOUS INHIBITORS

Column fraction	Proteolytic activity	Substrate preferentially cleaved	Approximate molecular weight	$PhCH_2SO_2F$	Chymostatin	EDTA	$CoCl_2$	Activated by
42	1	D	>500,000	++	+++	+++	+++	
45	2	A	>500,000	+++	−	−	++	
(50; 54)			(>500,000; 340,000)					
45	3	B	>500,000	(+)	++	−	+++	
46	4	C	>500,000	(+)	+++	(+)	++	
49	5	D	>500,000	−	++	+++	+	
51	6	B	500,000	(+)	(+)	−	++	EDTA
53	7	D	380,000	+	+++	+	+	
57	8	A,B,D	220,000	−	−	++	−	$CoCl_2$
(63; 67)			(110,000; 62,000)					
60	9	A	160,000	+	+	+++	−	$CoCl_2$
(65)		(A,B)	(82,000)					
64	10	D	95,000	+	+++	+++	−	$CoCl_2$
68	11	A	56,000	+++	(+)	+++	−	

Note. Tests were done with the fractions of the gel filtration experiment as outlined under Materials and Methods. Inhibitor concentrations were: $PhCH_2SO_2F$, saturated by addition of crystals; chymostatin, 0.25 mg/ml; EDTA, 1 mM; $CoCl_2$, 1 mM. Extent of inhibition is marked as: −, no inhibition; +, weak inhibition (approx up to 30%); ++, intermediate inhibition (approx 30–60%); +++, strong inhibition (approx 60–100%); (+) weak inhibition, possibly caused by overlapping specificities.

were found to be eluted from the Sephadex column. Two independent chromatography experiments gave identical results. All activities were completely inhibited by Hg^{2+} ions in a concentration of 0.2 mM. The discrepancy of this result with the finding in crude extracts, where Hg^{2+} ions had only partly inhibitory effects, may be explained by a protection of the proteolytic activities in the crude extract by other proteins, which are separated during chromatography.

Five proteolytic activities are found in the range of the void volume of the column so that their respective molecular weights cannot be determined under these conditions. Activity 1, which preferentially splits the substrate Suc-Ala-Ala-Pro-Phe-Nan, has the smallest elution volume. However, as overall activity measured with this substrate is generally low (Table I), activity 1 can be detected only after prolonged incubation (not shown in Figs. 1 and 2). This activity is completely inhibited by EDTA and chymostatin. It is also partly inhibited by $PhCH_2SO_2F$ and by Co^{2+} ions. Leaving the column with a slightly higher elution volume is activity 2, which mainly splits Bz-Pro-Phe-Arg-Nan. It is strongly inhibited by $PhCH_2SO_2F$ and exhibits a partial sensitivity to Co^{2+} ions. Bz-Ile-Glu-Gly-Arg-Nan cleavage is due to another hydrolytic activity (activity 3), which can be distinguished from activity 2 by its relative sensitivity to chymostatin. Co^{2+} ions strongly inhibit this activity, whereas EDTA and $PhCH_2SO_2F$ show no effect. Cbz-Gly-Gly-Leu-Nan is split almost exclusively by activity 4 (Fig. 1C). This activity is highly sensitive to chymostatin inhibition. Activity 5 can be visualized by its action on Suc-Ala-Ala-Pro-Phe-Nan. It is characterized by a strong inhibition by EDTA. $PhCH_2SO_2F$ has no effect on this activity.

Activity 6, which has a molecular weight near 500,000, splits Bz-Ile-Glu-Gly-Arg-Nan and can be activated about twofold

by EDTA. Co^{2+} ions reduce the activity of this enzyme to about 50%. Activity 7 with a molecular weight of about 380,000 preferentially hydrolyzes Suc-Ala-Ala-Pro-Phe-Nan. Chymostatin inhibits this activity to about 80%. Both EDTA and $PhCH_2SO_2F$ have weak inhibitory effects on this activity.

Proteolytic activities 8, 9, and 10 show a strong increase in activity when Co^{2+} ions are present. Activity 8 has an approximate molecular weight of 220,000 and is partly inhibited by EDTA. Its activity measured with substrate Bz-Ile-Glu-Gly-Arg-Nan is increased more than fourfold by addition of Co^{2+} ions. Activity 9 can be detected by its action on Bz-Pro-Phe-Arg-Nan. Activation by Co^{2+} ions is about fourfold. This enzyme has an approximate molecular weight of 160,000 and is completely inhibited by EDTA. Activity 10 splits Suc-Ala-Ala-Pro-Phe-Nan and is activated about twofold by Co^{2+} ions. It is strongly inhibited by EDTA and chymostatin. This activity has a molecular weight of about 110,000.

Activity 11 splits Bz-Pro-Phe-Arg-Nan. The approximate molecular weight is 56,000. It is completely inhibited by EDTA. $PhCH_2SO_2F$ reduces its activity to about 30%. Whether this inhibition pattern indicates the presence of two activities is unclear. In the molecular weight range below 50,000 two additional activities can be detected but their activity with the described substrates is too low to give a definite characterization.

As proteinases are able to form complexes with other cellular proteins (which may be their substrates) one and the same enzyme can possibly be eluted from the column at different elution volumes. Considering this possibility, activities with identical inhibition patterns were viewed as one proteolytic activity and were numbered after the activity exhibiting the smallest elution volume (Table II: Activities 2, 8, and 9). For the reason mentioned above the molecular weight determination of all proteolytic activities can only be preliminary. Another fact might also lead to a reduction in the actual number of new enzymes as compared to the number of proteolytic activities visible after gel chromatography. In some instances a concerted action of endoproteinase and aminopeptidase activity might be necessary for the liberation of 4-nitroaniline from the chromogenic peptide substrates. The proteinase and aminopeptidase would probably exhibit a different inhibition spectrum. In some column fractions activity of the endoproteinase is rate limiting while in others the aminopeptidase activity is rate limiting, thus the inhibition pattern will change across a single proteinase peak, leading to two or more activity peaks. Three aminopeptidases, which are sensitive to EDTA inhibition are known at present in *Saccharomyces cerevisiae* [(17, 18, 19); for reviews see (1, 2)]. Other difficulties in assigning the proteolytic activities observed after chromatography to single enzymes might reside in a different sensitivity of an enzyme to various inhibitors or activators with different substrates.

On the other hand, it is quite clear that gel filtration does not quantitatively separate activities which show relative small molecular-weight differences. So partial inhibition of an activity cleaving a certain substrate by two inhibitors of different action spectrum may actually be caused by two enzymes with overlapping specificities. Thus, these studies cannot give an exact number of new proteolytic enzymes detectable with the substrates employed. However, the existence of multiple new proteinases is evident.

The mutations in the structural genes of carboxypeptidase Y, carboxypeptidase S, and proteinase B lead to complete absence of the three enzyme activities (4–6, 10; D. H. Wolf and C. Ehmann, in preparation). If one were still to argue that the different activities separated by gel chromatography might be due to traces of proteinase B, carboxypeptidase Y, or carboxypeptidase S which might have remained in the triple mutant, this can be ruled out on the basis of the respective inhibition patterns. Proteinase B exhibits high activity against Suc-Ala-Ala-Pro-Phe-Nan (data not shown). When wild-type extract containing proteinase B is

chromatographed under the same conditions as mutant extract, proteinase B measured with azocoll as substrate is only visible after activation by sodium dodecyl sulfate, which dissociates the specific inhibitor protein I^B (4, 13), (data not shown). Sodium dodecyl sulfate activation is also required for detection of proteinase B activity against Suc-Ala-Ala-Pro-Phe-Nan. However, no activation of Suc-Ala-Ala-Pro-Phe-Nan splitting activity by sodium dodecyl sulfate was observed in crude extracts of the triple mutant. Furthermore, the main peak of proteinase B activity has an elution volume even greater than activity 11 (data not shown).

Only activity 2 shows an inhibition pattern similar to carboxypeptidase Y (1, 2), namely inhibition by $PhCH_2SO_2F$ and Hg^{2+} ions. In contrast to this new proteolytic activity, purified carboxypeptidase Y is eluted in the molecular-weight range of fraction 60 to 70 (data not shown). Only activity 8 shows an inhibition pattern comparable to carboxypeptidase S, that is, inhibition by chelating agents only. However, when cell extract containing wild-type carboxypeptidase S activity is chromatographed, the main enzyme activity appears with an elution volume near the exclusion volume (data not shown).

Known proteolytic enzymes, which are left at wild-type levels in the triple mutant are proteinase A, three aminopeptidases, and one dipeptidase (1, 2). None of the newly found activities can be due to proteinase A action, as they are insensitive to pepstatin inhibition (data not shown), a powerful inhibitor of proteinase A. Furthermore, proteinase A is active only at acidic pH and is insensitive to $PhCH_2SO_2F$ and EDTA inhibition (1, 2). Use of the chromogenic substrates blocked or carrying a D-amino acid residue at the α-amino-end of the peptide excludes aminopeptidase cleavage.

We not only searched for new soluble proteolytic activities but also looked for enzymes which, because of association with cellular structures, were precipitated after cell disintegration by centrifugation at 100,000*g*. As can be seen in Table III, of the 14 chromogenic peptide substrates tested, 4 show detectable cleavage by such a precipitated fraction without further addition. As shown in Table III, the proteinase activity against the chromogenic peptide substrates in such a membranous fraction is rather insensitive to inhibition by $PhCH_2SO_2F$, chymostatin, and EDTA. However, the enzyme activity which, because of its origin in the membrane fraction will tentatively be called proteinase M, is highly sensitive to inhibition by Zn^{2+} ions.

As no endogenous aminopeptidase activity may be present in a precipitated and washed membranous fraction, any proteolytic activity that cleaves at a site other than the 4-nitroanilide bond may be undetectable. Therefore, we added microsomal aminopeptidase to assays of the membranous fraction with chromogenic peptide substrates that were not hydrolyzed without aminopeptidase. The results are summarized in Table III. Considerable proteolytic activity in the membranous fraction against Bz-Phe-Val-Arg-Nan, H-D-Val-Leu-Lys-Nan, and H-D-Val-Leu-Arg-Nan can be visualized after inclusion of aminopeptidase. This adds another proteolytic activity to the newly detected enzymes. Because of its occurrence in the precipitable fraction, we will preliminarily call this enzyme proteinase P. As mentioned already, from the experiments presented no definite bond splitting specificity can be elaborated for the soluble multiple enzyme activities because of possible proteolytic attack of the chromogenic substrates at a site other than the nitroanilide bond, followed by endogenous aminopeptidase cleavage. Because of the obvious absence of aminopeptidase activity in the washed membranous fraction, a preliminary specificity can be assigned to proteinases M and P. Liberation of 4-nitroaniline by proteinase M from the substrates attacked points to hydrolysis of the Lys-Nan and Arg-Nan bonds by this enzyme. Proteinase P cannot liberate 4-nitroaniline from its substrates without adding aminopeptidase. This points to Leu-Lys as the splitting site in the peptide H-D-Val-Leu-Lys-Nan, and Leu-Arg as the split-

TABLE III

PROTEOLYTIC ACTIVITIES IN THE MEMBRANOUS FRACTION OF STRAIN BYS 232-14-23 (*α, prb 1, prc 1, cps 1*)

Peptide substrate	sp act[a]	Percentage activity remaining after addition of				sp act with addition of aminopeptidase[a]
		$PhCH_2SO_2F$	Chymostatin	EDTA	$ZnCl_2$	
Bz-Pro-Phe-Arg-Nan	0.4	100	105	108	16	—
Tos-Gly-Pro-Lys-Nan	<0.1	—	—	—	—	—
Tos-Gly-Pro-Arg-Nan	0.1	138	156	131	30	—
Bz-Ile-Glu-Gly-Arg-Nan	0.68	105	111	98	10	—
Bz-Phe-Val-Arg-Nan	<0.1	—	—	—	—	0.45
H-D-Phe-Pip-Arg-Nan	<0.1	—	—	—	—	<0.1
H-D-Val-Leu-Lys-Nan	<0.1	—	—	—	—	0.21
H-D-Val-Leu-Arg-Nan	<0.1	—	—	—	—	0.28
Cbz-Val-Gly-Arg-Nan	0.36	110	94	78	32	—
Bz-Asp-Trp-Arg-Nan	0.13	—	—	—	—	—
Suc-Ala-Ala-Ala-Nan	<0.1	—	—	—	—	<0.1
Cbz-Gly-Gly-Leu-Nan	<0.1	—	—	—	—	—
Suc-Ala-Ala-Pro-Phe-Nan	<0.1	—	—	—	—	—
Suc-Gly-Gly-Phe-Nan	<0.1	—	—	—	—	—

Note. Cells were grown into early logarithmic phase and the membranous fraction was prepared as outlined under Materials and Methods. Inhibitor concentrations were: $PhCH_2SO_2F$, saturated by addition of crystals; chymostatin, 0.25 mg/ml; EDTA, 1 mM; $ZnCl_2$, 0.1 mM. Assays were done as outlined. Activity without inhibitors is set 100%. When endoproteinase activity was measured by addition of aminopeptidase, 25 μg of microsomal leucine aminopeptidase (aminopeptidase M, Sigma A 7761) was added to the test solution.

[a] Specific activity given as (nmol × min^{-1} × mg^{-1}).

ting site in the peptide H-D-Val-Leu-Arg-Nan.

DISCUSSION

The studies on a triple mutant of *Saccharomyces cerevisiae* lacking proteinase B, carboxypeptidase Y, and carboxypeptidase S, clearly demonstrate the existence of multiple previously undetected proteolytic enzymes in this organism. A thorough investigation will be necessary to give further information on the exact number of enzymes and their characteristics. In some cases this investigation may turn out to be quite difficult, because some activities against the synthetic 4-nitroanilide substrates are quite small. Previous studies on the proteinase mutants available demonstrated a necessary function for carboxypeptidase S and the vacuolar carboxypeptidase Y in supplying amino acids for growth from exogenous peptides (6), and for the vacuolar proteinase B, vital for internal protein degradation to generate amino acids for new protein synthesis under starvation and differentiation conditions (5). These functions require rather unspecific proteolysis. A variety of vital cellular processes, which were suggested to depend on quite specific proteolytic action, probably outside the vacuole, is most likely not catalyzed by these proteinases [5, 6, 20, 21; for reviews see (1, 2)]. Some of the newly detected proteolytic activities may be possible candidates for these more specific proteinase actions. As a prerequisite, they are all active at neutral pH, ensuring possible activity in cellular compartments outside the vacuole. Exact cellular localization of the enzymes has to be elaborated in future studies. Two activities are found with the precipitable membranous fraction. Whether they serve a special function on a cellular membrane has to be elucidated.

For example, it was reported that recovery of mating type *a* cells of *Saccharomyces cerevisiae* from growth arrest by the mating hormone α factor is triggered by proteolytic cleavage of α factor (22, 23). As an important step during the inactivation of α factor endoproteolytic cleavage between Leu 6 and Lys 7 of the tridecapeptide was found. This cleavage most probably occurs at the cell surface (23). Proteinase P activity is a likely candidate for this function, because it cleaves the bond between Leu and Lys endoproteolytically and is found associated with the membranous fraction of disintegrated cells. Final answers to the question about the biological function of the newly detected proteinases awaits further biochemical and genetic studies.

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