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THE 45 kDa AND 27 kDa YEAST'S PROTEIN KINASES ARE NOT IMMUNOLOGICALLY RELATED**

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Two yeast casein kinase type-1 species of 45 kDa and 27 kDa (CK1) were purified to apparent homogeneity and used for investigation of their immunological affinity. Antisera against the two kinases were isolated; the antibody against the 45 kDa kinase did not react with the 27 kDa enzyme. The 27 kDa casein kinase was recognized only by its own antibody. The obtained data strongly suggest that the low molecular mass CK-1 is not a proteolytic product of the 45 kDa kinase species.

Casein kinases belong to a family of protein kinases which are insensitive to cyclic nucleotides, Ca^{2+} or phospholipids, and *in vitro* prefer casein and phosvitin over histone and protamines as phosphorylable substrates. Casein kinases are generally divided into two classes according to their

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subunit structure, catalytic properties and chromatographic behaviour. Type-1 casein kinases (CK-1) are monomeric and relatively small enzymes (20 - 50 kDa), not retained by DEAE-cellulose and utilizing only ATP. Type-2 casein kinases (CK-2) are oligomeric enzymes (120-200 kDa) which bind to DEAE-cellulose and can utilize GTP, in addition to ATP, as a phosphate donor [1- 3].

Two species of casein kinase type-1 from yeast have been isolated and characterized [4, 5]. They differ in molecular mass and amino-acid phosphorylation site specificity. The larger enzyme, of 45 kDa, phosphorylates serine residues in casein, while the minor, 27 kDa fraction is capable also of phosphorylating threonine [5]. Both these kinases have similar chromatographic properties at the early steps of purification. The 45 kDa kinase is always accompanied by the low molecular mass species. Meanwhile we have found high proteolytic activity localized in fractions containing both CK-1 species. It was reasonable to ask whether the minor CK-1 species was a separate enzyme or the proteolytic derivative of the 45 kDa kinase. The results obtained seem to indicate the lack of immunological similarity between those two investigated enzymes.

MATERIALS AND METHODS

Saccharomyces cerevisiae wild strain SKQ2N and ABYS1 [6], were cultivated and harvested, and cell-free extracts were prepared as described previously [7] with the exception that mechanical disintegration was replaced by spheroplastization of the yeast cells [8].

Purification of CK-1. Casein kinase type-1 from yeast was isolated and purified as described before [4]. In short, the postribosomal supernatant was precipitated with ammonium sulphate at 50% saturation and, after overnight dialysis against buffer containing 20 mM Tris/HCl, pH 7.5, 6 mM 2-mercaptoethanol, 0.5 mM EDTA, 0.5 mM PMSF was chromatographed on DEAE-cellulose column. The casein kinase activity was found in the flowthrough fractions. This activity was then rechromatographed on P-cellulose and on Heparin-Sepharose 4B. The applied procedure allowed for a good separation of two casein kinase 1 species. The final preparation of both enzymes, when tested by SDS/PAGE, appeared to be nearly homogeneous. During the whole CK-1 purification procedure 0.5 mM PMSF was used to inhibit protease activity.

Casein kinase assay. The activity of casein kinase was determined as described previously [9] by incubating 50 µl of a medium containing 30 µM [γ-^{32}P]ATP (sp. act. 1000 c.p.m./pmol), 15 mM $MgCl_2$, 6 mM 2-mercaptoethanol, 40 - 70 µg casein, 20 mM Tris/HCl, pH 7.5 with variable amounts of enzymes.

Polyacrylamide gel electrophoresis. Electrophoretic analyses of enzyme preparations and radiolabelled casein fractions were performed in the presence of 0.1% SDS on 10% or 12.5% polyacrylamide gel according to Laemmli [10]. The gels were stained with Coomassie brilliant blue R-250.

Yeast protease was isolated from cytosolic fraction of yeast strain SKQ2N when PMSF was omitted in the whole CK-1 purification procedure. After chromatography on DEAE-cellulose column the proteolytic activity was found in the fractions containing CK-1. Both activities were separated during P-cellulose chromatography. The protease was localized in the flowthrough fractions. It was concentrated using Amicon apparatus with UM-10 filter and chromatographed on the Sephacryl S-200 column (1 × 90 cm). Proteolytic activity towards casein and CK-1 was found in the void volume fractions. Enzyme was concentrated and used in the experiments with CK-1 proteolysis. The protease on SDS/PAGE showing 10 - 12 protein bands with molecular mass between 20 and 150 kDa. Similar high molecular mass proteases isolated from yeast have been reported previously [11].

Localization of proteolytic activity was performed in the mixture containing 20 mM Tris/HCl, pH 7.5, 6 mM 2-mercaptoethanol and 40 -70 µg of the casein or 1 - 2 µg of the CK-1 45 kDa as substrates. Samples after 10 min incubation at 30°C were electrophoresed through 12.5 % polyacrylamide gel in the presence of 0.1% SDS.

This proteolytic activity was found to be stimulated by SDS (0.02 - 0.05 %), Triton X-100 (0.1 %) and oleic acid (200 M) and strongly inhibited by PMSF and soybean trypsin inhibitor.

Immunodetection of casein kinase type-1. The appropriate kinase preparation (2 - 10 µg) was applied onto 10% SDS/PAGE. After electrophoresis proteins were blotted onto nitrocellulose paper (NEN, Dreiech, Germany) using a semidry blotting apparatus. The membrane and the blotting paper were soaked before the transfer in blotting buffer (25 mM Tris/HCl, pH 8.3, 192 mM glycine). After the 90 min of transfer at 300 mA the blot was blocked for 60 min in 1% non-fat dry milk in Tris-buffered saline (10 mM Tris/HCl, pH 7.4, 0.9% NaCl) followed by incubation with

diluted 500 - 1000 fold an antibody raised in rabbit [12]. Incubation with the first antibody took 60 min at room temperature and was followed by washing the membrane three times for 10 min in Tris-buffered saline containing 0.1% Tween 20. Incubation with a phosphate-conjugated goat anti-rabbit IgG (Medac, Hamburg, Germany), used as a second antibody, was for 60 min at room temperature. This antibody was diluted 1000-fold in 1% milk/Tris-buffered saline. Then the membrane was washed three times for 10 min in Tris-buffered saline/Tween 20 and then equilibrated in AP-buffer (0.1 M Tris/HCl, pH 9.5, 0.1 M NaCl, 5 mM $MgCl_2$). Finally the membrane was stained with NBT (*p*-nitro-tetrazolium blue) and BCIP (5-bromo-4-chloro-3-indolyl phosphate) as described earlier [13].

Affinity purification of CK1 antibody. CK-1 (10 μg) was analyzed on 12.5% SDS/PAGE and blotted onto nitrocellulose paper. After blotting the membrane was stained with Ponceau Red to visualise the transferred kinase. The stained CK-1 bound was excised from membrane, destained in water and then incubated overnight at 4°C in 100 μl anti-CK-1 45 kDa or 27 kDa antiserum, respectively. The membrane was washed three times with Tris-buffered saline/Tween 20 and incubated for 1 min in 3 ml of buffer containing 100 mM glycine/HCl, pH 2.2, 20 mM $MgCl_2$, 5 mM KCl. The membrane was transferred to a syringe and the glycine solution was forced twice through the paper. The eluted material was neutralized by addition of 1 mM Tris base (0.3 ml). After addition of 1 ml Tris-buffered saline the solution was concentrated using a stirred Amicon ultrafiltration apparatus with a UM-10 filter. The concentrated material was dialysed overnight against Tris-buffered saline.

RESULTS AND DISCUSSION

It has been previously demonstrated that yeast cells contain several protein kinases phosphorylating casein and some ribosomal proteins. One of the enzymes, casein kinase type-1, was accompanied at the early steps of purification by another kinase, belonging to the same type of casein kinases [4].

When casein kinases were purified from the wild type of *Saccharomyces cerevisiae* in the absence of protease inhibitors a high proteolytic activity towards casein and the 45 kDa CK1 was found. This protease activity was significantly enhanced by low concentration of SDS, Triton X-100 or oleic

acid. A similar proteolytic activity was purified from yeast [11] and cytosolic fraction of rat liver [14]. The yeast protease was found to be very sensitive to PMSF. In the presence of 0.5 mM PMSF the proteolytic activity was completely inhibited (not shown). Based on the above observations, the protease isolated from yeast cytosol and exogenous trypsin were tested to compare their ability to cleave of the 45 kDa kinase. As shown in Fig. 1 in both cases two major proteolysis products 39 kDa and 27 kDa were observed.

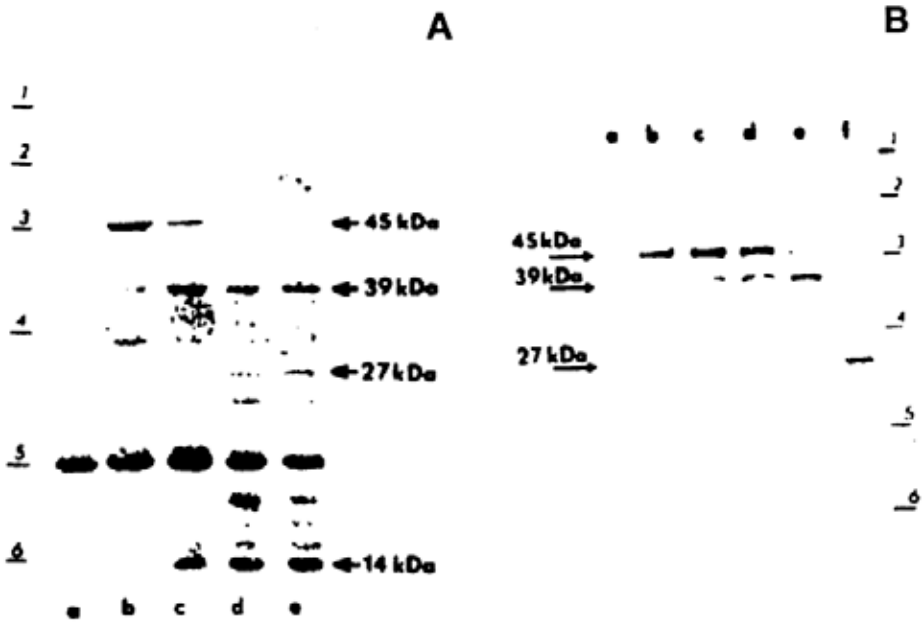

Fig. 1. Proteolytic digestion of the 45 kDa casein kinase with trypsin (A) and protease isolated from yeast (B). The homogeneous 45 kDa CK1 (2 μg) was digested with 0.1 μg of trypsin (A) or 1 μg of yeast protease (B) [tracks (a)]. Proteolysis was carried out for: 0 (b), 5 (c), 15 (d), 30 (e), or 60 (f) min at room temperature. The reaction was stopped by addition of 2 μg of soybean trypsin inhibitor (A) or PMSF to a final concentration of 2 mM (B). Electrophoresis was carried out as described in Materials and Methods in the presence of the following molecular mass standards: 1) phosphorylase b (94 kDa), 2) bovine serum albumin (67 kDa), 3) ovalbumin (43 kDa), 4) carbonic anhydrase (30 kDa), 5) soybean trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa).

Enzymatic activity of the digested 45 kDa kinase against casein was also tested (Fig. 2). When αS1-casein and whole casein were used as protein

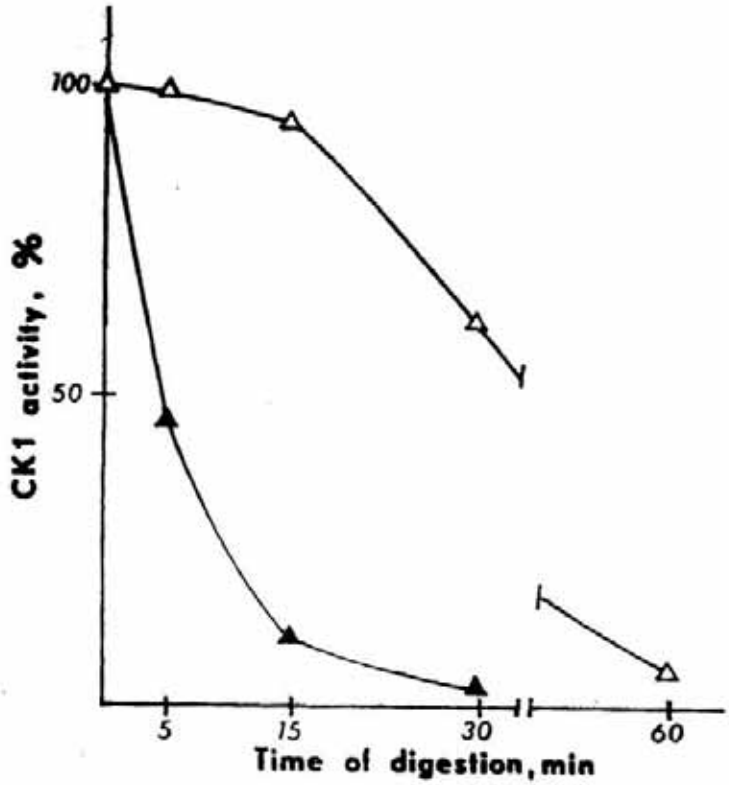

Fig. 2. Effect of proteolysis on the 45 kDa casein kinase activity. The 45 kDa CK1 (2 μg) was digested with 1 μg of protease isolated from yeast (open triangles) or 0.1 μg of trypsin (closed triangles). Proteolysis was stopped by addition of PMSF to a final concentration of 2 mM and the kinase digest was examined in a phosphorylation reaction with total casein as a protein substrate as in Material and Methods section

substrates (during the experiments shown in Fig. 2), the only amino-acid phosphorylated by the 45 kDa kinase and their proteolytic digest was serine (not shown). As we have earlier mentioned [5] this is in contrast with our previous observations showing also the phosphorylation of threonine by the low molecular mass CK-1.

It was of interest to ask whether there is any immunological affinity between the two investigated species of CK-1 and the proteolytic products (of 39 kDa and 27 kDa) of the 45 kDa casein kinase. To answer this question, antibodies against the 45 kDa and the 27 kDa enzyme were prepared. The antisera were tested against both kinase preparations and the 45 kDa kinase proteolytic products obtained with yeast protease or trypsin using the Western blotting technique. As can be seen in Fig. 3, the 45 kDa antiserum

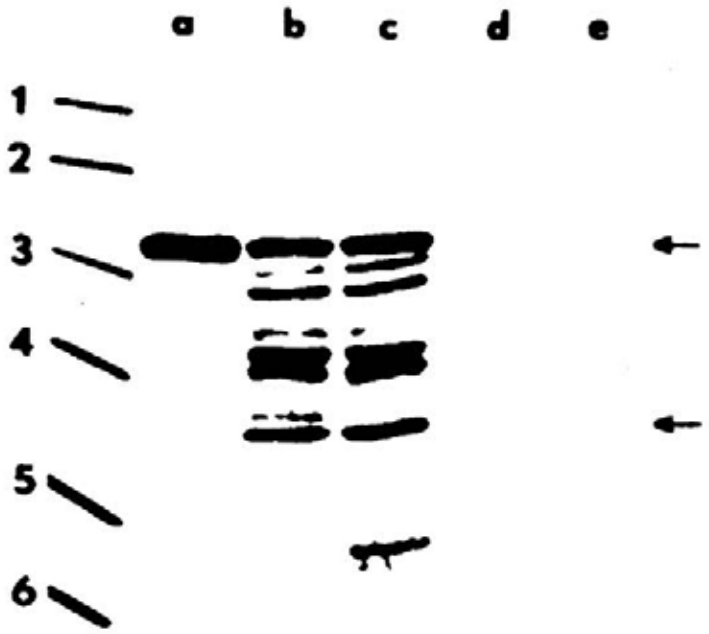

Fig. 3. The immunoblotting reaction of the anti-45 kDa CK-1-antibody with the yeast kinase type 1. The numbers represent protein standards as in Fig. 1. Tracks show the immunoreaction of the anti-45 kDa CK-1-antibody with: a, 2 μg of the 45 kDa CK-1 (control); b, 2 μg of the 45 kDa CK-1 digest obtained after 15 min hydrolysis with 1 μg of yeast protease; c, as "b" except 30 min hydrolysis; d, 2 μg of the 27 kDa CK-1 (control); e, 1 μg of the yeast protease preparation. Arrows show the positions of kinase preparations after 10% SDS/PAGE separation.

recognizes only the major kinase band and its proteolytic products. The minor casein kinase species did not react with this antiserum. When the 27 kDa kinase antiserum was used in the same experiments (Fig. 4) only the 27 kDa kinase was recognized.

The effect of antibodies on the activity of casein kinases was tested in the presence of IgG fractions (not shown). The obtained IgG fractions effectively inhibited only the activity of the 45 kDa kinase when antibody against this enzyme was used, and activity of the 27 kDa kinase was inhibited only in case of the 27 kDa antibody.

The observed lack of immunological affinity argues against the possibility that the minor species of yeast casein kinase type-1 is formed by proteolysis of the 45 kDa casein kinase.

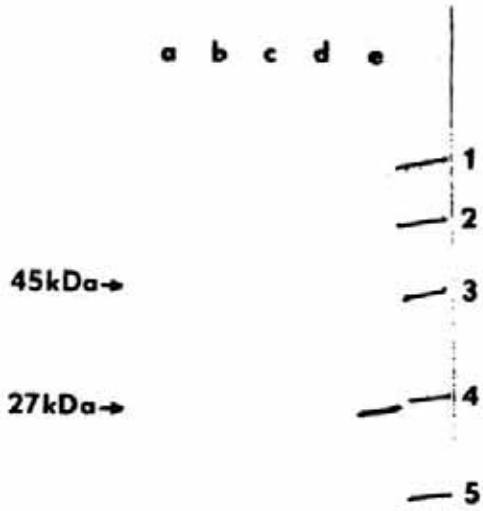

Fig. 4. The immunodetection of the type-1 yeast casein kinase with the anti-27 kDa CK-1-antibody. The numbers represent protein standards. Tracks show the immunoreaction of the anti-27 kDa CK-1-antibody with: a, 1 μg of the yeast protease; b, 2 μg of the 45 kDa CK-1; c, and d, 2 μg of the 45 kDa CK-1 digest after 15 and 30 min hydrolysis, respectively, with 1 μg of the yeast protease; e, 2 μg of the 27 kDa CK-1

Enzymes with the same properties as the ones shown here, were also obtained from yeast strain ABYS1 lacking the major vacuolar proteinases [6] (not included).

The results presented here strongly suggest that the 27 kDa casein kinase found in yeast is distinct from the major species of CK-1.

Enzyme activities corresponding to the casein kinase reported here were isolated also from baker's yeast [15] and *Dictyostelium discoideum* [16], but not from any higher organism.

Little is known about the *in vivo* functions of the reported enzymes, and how their activities are regulated during the cell cycle. To answer these questions a further search for the cellular substrate proteins phosphorylated by the discussed enzymes is needed.

REFERENCES

1. Hathaway, G. M. & Traugh, J. A. (1982) Casein kinases – multipotential protein kinases. *Curr. Top. Cell. Regul.*, **21**, 101 - 127.

2. Edelman, A. M., Blumenthal, D. K. & Krebs, E. G. (1987) Protein serine/threonine nases. *Ann. Rev. Biochem.*, **56,** 567 - 613.

3. Hunter, T. (1987) A thousand and one protein kinases. *Cell*, **50**, 823 - 829.

4. Szyszka, R., Kudlicki, W., Grankowski, N. & Gąsior, E. (1985) A minor species of a type I casein kinase from yeast phosphorylating threonine residues of protein substrate. *Biochim. Biophys. Acta*, **838**, 171 - 174.

5. Donella-Deana, A., Grankowski, N., Kudlicki, W., Szyszka, R., Gąsior, E. & Pinna, L.A. (1985) A type I casein kinase from yeast phosphorylates both serine and threonine residues of casein. Identification of phosphorylation sites. *Biochim. Biophys. Acta*, **829,** 180 - 187.

6. Emter, O. & Wolf, D. H. (1984) Vacuoles are not the sole compartments of proteolytic enzymes in yeast. *FEBS Lett.*, **166**, 321 - 325.

7. Kudlicki, W., Szyszka, R. & Gąsior, E. (1984) A cytoplasmic, cyclic nucleotide-independent casein kinase II from *Saccharomyces cerevisiae*. *Biochim. Biophys. Acta*, **784**, 102 - 107.

8. Szyszka, R. & Gąsior, E. (1984) Phosphorylation of ribosomal proteins during differentiation of *Saccharomyces cerevisiae*. *Acta Biochim. Polon.*, **31**, 375 - 382.

9. Kudlicki, W., Szyszka, R., Paleń, E. & Gąsior, E. (1980) Evidence for a highly specific protein kinase phosphorylating two strongly acidic proteins of yeast 60S ribosomal subunit. *Biochim. Biophys. Acta*, **633**, 376 - 385.

10. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head bacteriophage T4. *Nature (London)*, **227**, 680 - 685.

11. Achstetter, T., Emter, O., Ehmann, C. & Wolf, D. H. (1984) Proteolysis in eucaryotic cells. *J. Biol. Chem.*, **259**, 13334 - 13343.

12. Grankowski, N., Szyszka, R. & Pilecki, M. (1987) Immunological properties and peptide mapping of two type I casein kinases from yeast. *Acta Biochim. Polon.*, **34**, 45-49.

13. Grankowski, N. & Issinger, O. G. (1990) Subcellular localization of casein kinase I. *Biochem. Biophys. Res. Commun.*, **167**, 471 - 476.

14. Yamamoto, T., Nojima, M., Ishiura, S. & Sugita, H. (1986) Purification of the two forms of the high-molecular-weight neutral proteinase ingensin from rat liver. *Biochim. Biophys. Acta*, **882**, 297 - 307.

15. Sternbach, H. & Künzel, H. (1987) Purification of a yeast protein kinase sharing properties with type I and type II casein kinases. *Biochemistry*, **26,** 4207 - 4212.

16. Ospina, B. & Fernandez-Renart, M. (1990) Characterisation of three casein kinases type I from *Dictyostelium discoideum*. *Biochim. Biophys. Acta*, **1052,** 483 - 488.