mercaptoethylamine activated strongly and SH reagents inhibited drastically the enzymatic activity. Bivalent metals were inhibitory and E-600 (diethyl-*p*-nitrophenyl phosphate) and DFP (di-isopropyl fluorophosphate) had no influence even when a pre-incubation of 4 h was allowed. Molecular weight determinations using gel filtration on Sephadex G-100 [2] with albumin, 70 000,[5] and trypsin 23 800,[6] as standards gave a value of 48 000.

*Discussion.* A 140-fold purification was obtained by the method used. The purified enzyme is clearly a protease with a substrate specificity resembling that of bovine trypsin. Its pH optimum, reaction to a number of modifier substances, particularly sulphydryl reagents, as well as its different behaviour during the purification procedure show that the protease is not identical with either salivain [2] or glandulain.[7] The dependence on SH-groups, preference for BAA and BANA before BAPA [8] as substrate, the relatively slow hydrolysis of ester substrates,[9] and the molecular weight of similar order [10] are features resembling those of cathepsin B. A marked contrast is the neutral pH optimum of the protease (reported here) compared with the pH 5.0—5.5 optimum of cathepsin B.[11] The presence of enzymes, resembling closely the submandibular protease has been demonstrated in other tissues, *e.g.* in thyroid.[12] Also, Curoff [13] has purified from rat brain tissue (32-fold) a neutral SH-dependent protease with characteristics resembling those of the submandibular protease. The possible identity of these enzymes remains to be decided after further purification and characterization of the brain protease has been carried out.

1. Riekkinen, P. J. and Hopsu, V. K. *Ann. Med. Exptl. Biol. Fenniae, (Helsinki)* **43** (1965) 6.
2. Riekkinen, P. J., Ekfors, T. O. and Hopsu, V. K. *Biochim. Biophys. Acta* **118** (1966) 604.
3. Riekkinen, P. J., Ekfors, T. O., Hollmén, T. and Hopsu, V. K. *Enzymologia. In press.*
4. Venkataraman, A. *J. Biol. Chem.* **173** (1948) 641.
5. Andrews, P. *Biochem. J.* **91** (1964) 222.
6. Desnuelle, P. In Boyer, P. D., Lardy, H. and Myrbäck, K. (Eds.), *The Enzymes*, Academic, New York — London, Vol. 4, p. 124.
7. Riekkinen, P. J., Ekfors, T. O. and Hopsu, V. K. *Enzymologia. In press.*
8. Nagel, W., Filling, F., Peschke, W. and Schmidt, F. H. *Z. Physiol. Chem.* **340** (1965) 1.
9. Greenbaum, L. M. and Fruton, J. S. *J. Biol. Chem.* **226** (1957) 173.
10. Lundblad, G. and Falksveden, L. G. *Acta Chem. Scand.* **18** (1964) 2044.
11. Greenstein, J. P. and Leuthardt, F. M. *J. Natl. Cancer Inst.* **8** (1947—48) 77.
12. Suominen, J. and Hopsu, V. K. *Data to be published.*
13. Curoff, G. *J. Biol. Chem.* **239** (1964) 149.

Received May 10, 1966.

## The Occurrence of Amino Acid Naphthylamidase in Baker's Yeast

ANDERS TJEDER

*Institute of Biochemistry, University of Uppsala, Uppsala, Sweden*

Yeast contains proteolytic enzymes which hydrolyze various proteins and peptides. These enzymes are described in early works by Dernby [1] and by Grassmann, Willstätter, and co-workers.[2-5] Besides proteinase and dipeptidase activity, an amino polypeptidase hydrolyzing DL-leucinamide and various peptides is reported. Another polypeptidase has been purified by Johnson [6] from brewer's bottom yeast. The proteinases in baker's yeast have been studied by Lenney,[7] but his work does not concern exopeptidases.

I have found in extracts of commercial baker's yeast, (*Saccharomyces cerevisiae*), an enzyme which hydrolyzes several amino acid naphthylamides. These chromogenic substrates are often used for the assay of leucine aminopeptidase, although Smith and Hill [8] have called attention to the possibility that these compounds might be hydrolyzed also by other enzymes. No hydrolysis of L-leucinamide, a classical substrate for leucine aminopeptidase determination, was detected with the yeast extract. The enzyme studied might therefore be called an amino acid naphthyl-

amidase or, more generally, an arylamidase.

Fresh commercial baker's yeast was disintegrated by a dry ice procedure described earlier by v. Hofsten and Tjeder.[9] Approximately 275 ml extract, pH 5.6—5.7, containing about 10 % solids was obtained from 1000 g yeast cake. The hydrolysis of L-leucyl-β-naphthylamide-HCl (LNA) and a number of similar arylmides (obtained from Dr. Theodor Schuchardt, München) was determined according to the procedure of Goldbarg and Rutenburg [10] using a substrate concentration of $3 \times 10^{-4}$ M in $5 \times 10^{-3}$ M Tris-HCl, pH 8.0. One unit of enzyme activity is defined as that amount which causes the hydrolysis of one μmole of substrate per min at 25°. The cell-free yeast extract was found to contain 450—750 milliunits of LNA-hydrolyzing activity per ml. Centrifugation of the extract for 90 min at 105 000 × *g* in a Spinco Model L preparative ultracentrifuge caused no loss in activity, which shows that the enzyme is present in a soluble form. No hydrolysis of L-leucinamide-HCl was detected with the extract, by the titrimetric method of Grassmann and Heyde.[11] The method was checked with other enzyme material containing leucine aminopeptidase. The yeast extract also contains an enzyme which hydrolyzes β-naphthylacetate. This esterase activity was determined according to Nachlas and Seligman.[12]

The arylamidase is most stable in the pH range 7—8. The yeast extract could be frozen and thawed and could be stored frozen at −26° for several weeks without any decrease in arylamidase activity. On storing the extract at 5° with 2 % butanol to prevent bacterial growth the arylamidase activity decreased slowly. Precipitating the arylamidase with ammonium sulphate and redissolving it caused a 50 % loss of activity. Dialysis of yeast extract against 0.01 M EDTA in 0.1 M Tris-HCl, pH 8.0 for 21 h lowered the arylamidase activity to 35 % of its original value, while dialysis against distilled water for the same time did not lower the activity. Addition of $Ba^{2+}$ to the extract at a concentration of 0.01 M increased the arylamidase activity by 75 %. $2.5 \times 10^{-3}$ M $Ca^{2+}$, $Co^{2+}$, $Mg^{2+}$, or $Mn^{2+}$ showed little or no effect.

When the extract was subjected to gel filtration on Sephadex G 100 the arylamidase activity appeared in a symmetrical peak after the passage of approximately 1.7 void volumes, as shown in Fig. 1. Activity against the β-naphthylamides of glycine (GNA), DL-alanine (ANA), and L-leucine was determined in the eluate. ANA was hydrolyzed at the same rate as LNA, but the activity against GNA was about five times lower. No separation of these activities was achieved by gel

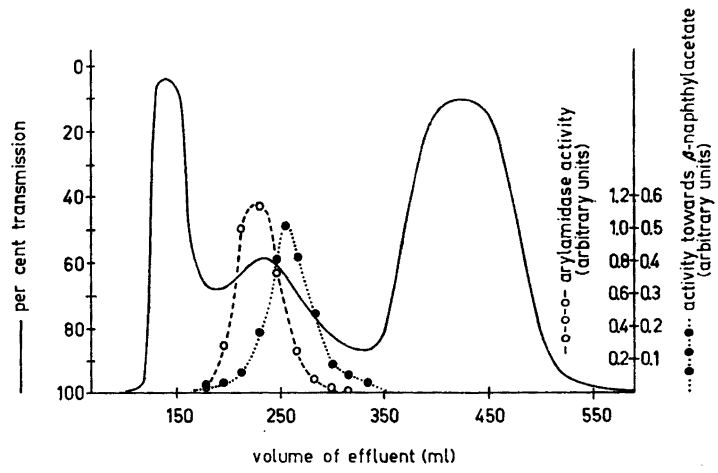


*Fig. 1.* Gel filtration of yeast extract. Sephadex G 100, equilibrated with 0.1 M Tris-HCl buffer, pH 8.0 was used in a glass column 3.5 cm in diameter and 48 cm long. The void volume was 125 ml. Elution was carried out at 5° with the same buffer. The UV-curve was obtained with the aid of a LKB Uvicord at 254 mμ.

filtration. A number of fractions also contained esterase activity, but this enzyme was eluted in a peak which was distinctly more retarded.

The pH-optimum for the hydrolysis of LNA was determined in a Tris-HCl buffer system, 0.05 M in Tris. The enzyme material used for the determination was partially purified from the yeast extract by means of gel filtration. The curve in Fig. 2 shows the enzyme activity *versus* pH.

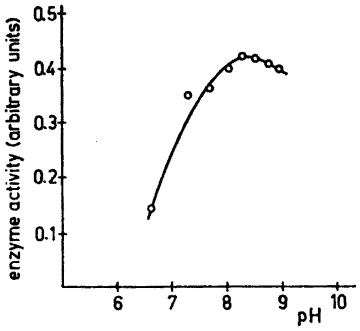


*Fig. 2.* pH dependence for hydrolysis of LNA by arylamidase from baker's yeast.

After partial purification by gel filtration the arylamidase was less stable than in the raw extract. It could still be frozen and thawed without loss of activity, but when the purified material was stored for ten days at −26° the arylamidase activity decreased by as much as 50 %. On storage at 5° the activity declined more rapidly than in unfractionated material. The recovery in gel filtration was 40−70 %.

Zone electrophoresis [13] of arylamidase, partially purified by gel filtration, was carried out on cellulose in sodium phosphate buffer, pH 6.5, ionic strength 0.05. The electrophoresis was run on a column (1.3 cm in diameter and 51 cm long) for 18.5 h at 700 V, (15 mA). Under these conditions the arylamidase did not move appreciably. No separation of the activities towards the different amino acid naphthylamides tested was observed. This and the result from gel filtration indicate that the same enzyme hydrolyzes the different amino acid naphthylamides. The further purification achieved through electrophoretic removal of impurities was nullified by the decline of enzymatic activity which occurred during electrophoresis. The recovery was only 20 %.

The biological significance of the arylamidase remains to be determined. Its natural substrate is unknown, and detailed specificity studies must be postponed until the enzyme is obtained in pure form.

*Acknowledgements.* The author is indebted to Professor Arne Tiselius, Drs. Bengt v. Hofsten, and Jerker Porath of this institute, for valuable help in different forms, and to Dr. David Eaker for linguistic revision of the manuscript.

1. Dernby, K. G. *Biochem. Z.* **81** (1917) 107.
2. Willstätter, R. and Grassmann, W. *Z. physiol. Chem.* **153** (1926) 250.
3. Grassmann, W. *Z. physiol. Chem.* **167** (1927) 202.
4. Grassmann, W. and Dyckerhoff, H. *Z. physiol. Chem.* **179** (1928) 41.
5. Grassmann, W. and Dyckerhoff, H. *Ber.* **61** (1928) 656.
6. Johnson, M. J. *J. Biol. Chem.* **137** (1941) 575.
7. Lenney, J. F. *J. Biol. Chem.* **221** (1956) 919.
8. Smith, E. L. and Hill, R. L. In Boyer, P.D., Lardy, H. and Myrbäck, K. (Eds.), *The Enzymes*, Academic, New York 1960, Vol. 4, p. 37.
9. Hofsten, B.v. and Tjeder, A. *J. Biochem. Microbiol. Technol. Eng.* **3** (1961) 175.
10. Goldbarg, J. A. and Rutenburg, A. M. *Cancer* **11** (1958) 283.
11. Grassmann, W. and Heyde, W. *Z. physiol. Chem.* **183** (1929) 32.
12. Nachlas, M.M. and Seligman, A. M. *J. Biol. Chem.* **181** (1949) 343.
13. Porath, J. *Biochim. Biophys. Acta* **22** (1956) 151.

Received June 9, 1966.