Short Communication

INHIBITION OF PROTEINASE B AND STABILISATION OF MALATE DEHYDROGENASE IN EXTRACTS OF THE YEAST *SACCHAROMYCES CEREVISIAE* BY TURKEY EGG WHITE FRACTION II-T

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The presence of proteolytic enzymes in yeast extracts has been known for a long time, and to date over 40 enzymes have been detected although not all have been characterised (1). Some of these enzymes pose significant problems in the study of yeast biochemistry through their proteolytic action in extracts and during experimental manipulations. Problems encountered include loss of protein yield, instability and creation of artefacts (18). For example, malate dehydrogenase is so unstable in yeast extracts due to proteolytic attack that purification only became possible when the serine protease inhibitor phenylmethanesulphonylfluoride (PMSF) was added to the extracts. Alternatively, the action of proteases can result in production of modified forms of an enzyme which still retain catalytic activity but gives the appearance of the presence of isoenzymes. Yeast hexokinase provides a good example of this phenomenon which has been known for many years (19). More recently, it has been shown that the small s-monomer of yeast glycogen phosphorylase is an artefact due to the action of proteinase A (PrA). Mutants lacking this protease do not have any of the s-monomer and addition of the PrA inhibitor, pepstatin, to extracts of wild-type cells also results in the absence of the small protein (26).

The vacuolar proteases seem to cause the main problems. Of these, proteinase B (PrB) is widely regarded as the most significant due to its pH optimum around 7 and its characteristic activation by heat and SDS, conditions which are often found in biochemical work (9,22). It is accepted that elimination of PrB activity reduces problems associated with proteases and use of inhibitors such as PMSF is

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now routine in certain types of work (10). However, inhibitors such as PMSF and DFP are toxic reagents which can pose significant hazards (18). PMSF does not readily dissolve in water and organic solvents must be used to dissolve the compound in the first instance. It has a half-life of only 100 min at pH 7 with hydrolysis occurring even more quickly at higher pH values. In some situations, re-addition of the inhibitor is necessary at intervals throughout the experimental procedure. Thus, there is a clear need for more stable and less dangerous inhibitors for routine use.

Avian egg white proteins are members of a large and diverse group of compounds which have long been known to inhibit proteolytic enzymes (4,16). The best known inhibitor of this type is derived from chicken egg white and its action against bovine trypsin has been well documented (5,20,25). Action against yeast PrB was noted during a study on purification of the enzyme (11). Turkey egg white has been reported to inhibit Subtilisin (15) which is a serine protease with a high degree of homology with PrB (17). As turkey egg white is available at much lower cost than chicken egg white preparations, the effect of turkey egg white on serine proteases was investigated to see if it has potential as an economical and innocuous inhibitor.

A polyploid, brewing strain of the yeast *Saccharomyces cerevisiae* NCYC 1108 was used throughout. Cells were maintained on Sabouraud dextrose agar slopes at 4°C and grown for experimental purposes in batches of 100 ml medium (glucose 4%, yeast extract 1%) at 25°C for 48 h. To prepare yeast extracts, cells were collected by centrifugation at $2,000 \times g$ for 10 min at 4°C. They were washed twice with K phosphate buffer, 0.1 M pH 7.4, and resuspended in 10 ml of the same buffer. This suspension was passed through an SLM-Aminco French Cell Press three times at 1,200 psi. The extract was clarified by centrifugation at $4,000 \times g$ for 15 min at 4°C, after which 8 ml was removed and mixed with 30 ml distilled water containing penicillin G K salt to give a final antibiotic concentration of 10^5 U per litre. Activation of PrB and carboxypeptidase Y (CpY) in cell-free extracts (6-8,12-14,23) was effected by adjustment of the pH to 5.0 with 0.2 M acetic acid followed by incubation for 24 h. Earlier work (23) showed that, with the yeast strain used in this paper, PrA was easily detectable in the initial extract, there was a trace of CpY activity but PrB could not be detected. After incubation at pH 5 there was no change in PrA activity, CpY increased about three fold and PrB was easily measurable. Use of the PrA inhibitor, pepstatin, reduced the rate of activation by more than half so PrA is clearly an important component of the activation system. Once activated, PrB can also contribute to the activation of its own pro-enzyme and that of CpY but the rate of reaction is low because of the unfavourable pH. Autoproteolysis in yeast extracts was measured by the addition of 10% cold trichloroacetic acid after an appropriate period of incubation followed by measurement of A_{280} of the supernatant. PrA was assayed using acid-denatured haemoglobin as substrate (21), PrB with azocoll (3) and CpY with the chromogenic peptide BTPNA (2). Other proteases were purchased from Sigma, St. Louis, MO, U.S.A.

and assayed according to the manufacturer's instructions. Malate dehydrogenase was assayed spectrophotometrically at pH 9.2 in diethanolamine buffer (24). An incubation period of 25 min was incorporated after mixing yeast extract and NAD to allow for the action of dehydrogenases on endogenous substrates in the extract before adding the Na malate (0.25 ml, 250 mM, pH 9.2) to start the assay. Of the materials used, Azocoll, BTPNA, bovine haemoglobin, lactic acid, penicillin G K salt, turkey egg white type II-T, L-malic acid, diethanolamine, and NAD were obtained from Sigma, dimethylformamide from Prolabo, France, yeast extract, mycological peptone and Sabouraud dextrose agar from Unipath Ltd. (Oxoid), Basingstoke, Hampshire, England. All other reagents were from BDH, Poole, Dorset, England.

The effect of turkey egg white on a range of proteases with different specificities was measured. Standard assays were used and when the egg white preparation was included at 15 μ g per milliliter, trypsin was inhibited by 95%, chymotrypsin and yeast PrB by 84%. No effect was found on any of the other proteases tested: yeast PrA, yeast CpY, *Staphylococcus aureus* protease, ficin, bromelain, *Aspergillus* acid protease, pepsin, thermolysin, and *Bacillus polymyxa* protease. It appears that the turkey egg white is effective against serine proteases alone of the major types of protease. When yeast PrB was assayed with inclusion of turkey egg white at a range of concentrations, the activity was halved by turkey egg white at 5 μ g per milliliter and eliminated at 42 μ g per milliliter. This compares favourably with the action of known protease inhibitors; to achieve 100% inhibition of PrB, PMSF is needed at 1 mM (174 μ g per milliliter) (11).

The effect of turkey egg white on stability of proteins in yeast extract adjusted to pH 5.0 was determined. The extract was divided into portions, turkey egg white added at a range of concentrations from 0.1 to 1.0 mg per milliliter and then activation was carried out by incubation at 25°C. On the basis of the release of TCA-soluble peptides, turkey egg white significantly reduced the amount of proteolysis during the incubation; 300 μ g per milliliter caused almost 70% reduction in proteolysis, the maximum effect found. This effect was confirmed by FPLC gel permeation analysis which showed that turkey egg white and PMSF caused the same degree of stabilisation of molecular weight profiles in extracts adjusted to pH 7.0.

To determine the effect of turkey egg white on the stability of malate dehydrogenase, extract was prepared and the pH adjusted to 5.0. It was then centrifuged at $100,000 \times g$ for 10 min at 4°C to obtain an optically clear solution. The extract was divided into two portions and turkey egg white added to one to give a concentration of 250 μ g per milliliter. After 24 h incubation at 25°C to allow activation of PrB and CpY, each sample was adjusted to pH 7.0 and the malate dehydrogenase activity assayed at intervals over the next three days. The results in Fig. 1 show that, during the first 24 h, the presence of egg white had no effect. This was as expected as the egg white protein was shown earlier in this paper to have no effect on PrA, the protease most active at pH 5. After adjustment to pH 7.0, which

Fig. 1. Malate dehydrogenase activity in yeast extracts.

Yeast extracts, pH 5.0, were incubated at 25°C in the presence (○) and absence (□) of turkey egg white for 24 h to allow the native PrA to activate PrB and CpY. The pH was adjusted to 7.0 to allow action of PrB and CpY but prevent further PrA action. The malate dehydrogenase activity is given as the increase in A_{340} per minute under the standard assay conditions described in the text.

is the optimum for PrB activity, the malate dehydrogenase activity in the extract with no turkey egg white fell steadily over the course of the experiment. When turkey egg white was present, no change in malate dehydrogenase was found at pH 7.0. After 190 h, no malate dehydrogenase activity could be detected in the untreated extract. In contrast, there was still no loss of the enzyme in the presence of turkey egg white. As the egg white has been established to have no effect on CpY, the results can be explained entirely in terms of inhibition of PrB.

From a practical point of view, the results suggest that turkey egg white, or a fraction obtained from it, has potential as a routine inhibitor to be included in yeast extracts when damage is suspected as a result of PrB-catalysed proteolysis. The egg white material has several advantages over the protease inhibitors currently used in that it has no toxic or irritant properties and dissolves readily in aqueous buffers to form solutions which are stable at 4°C for at least 24 h.

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