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# The susceptibility towards proteolysis of intermediates during the renaturation of yeast phosphoglycerate mutase

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The renaturation of the tetrameric enzyme phosphoglycerate mutase from baker's yeast after denaturation in guanidinium chloride was studied. Three proteinases (trypsin, chymotrypsin and thermolysin) cause extensive loss of activity of samples taken during the early stages of refolding. As judged by SDS/polyacrylamide-gel electrophoresis, the proteinases cause substantial degradation of the polypeptide chain with no evidence for large quantities of fragments of $M_r$ greater than 6500. These data suggest that the early intermediates in the refolding, especially the folded monomer, possess a number of sites that are susceptible to proteolysis.

## INTRODUCTION

The renaturation of oligomeric enzymes after denaturation by agents such as guanidinium chloride has proved to be a useful experimental model for the folding and association of these enzymes during biosynthesis (Jaenicke, 1984). As shown by Hermann *et al.* (1981), the kinetic aspects of the re-association process and the catalytic properties of intermediates can be explored by comparing the rates of regain of enzyme activity and of quaternary structure, e.g. by the glutaraldehyde cross-linking technique. In most cases, the activity of intermediates is small, if not zero (Jaenicke, 1982; Jaenicke & Rudolph, 1983), although exceptions have been reported in the cases of rabbit muscle fructose bisphosphate aldolase (Chan *et al.*, 1973; Rudolph *et al.*, 1977) and rabbit muscle creatine kinase (Grossman *et al.*, 1981). In earlier work (Hermann *et al.*, 1983, 1985) we studied some aspects of the reconstitution of the tetrameric enzyme phosphoglycerate mutase (EC 2.7.5.3) from baker's yeast. Over a range of concentrations, the re-association process could be described by a model of the type:

$$4M \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} 2D \overset{k_{+2}}{\rightarrow} T \qquad (1)$$

where M, D and T represent monomer, dimer and tetramer respectively and where the values of $k_{+1}$, $k_{-1}$ and $k_{+2}$ are $6.26 \times 10^3$ $M^{-1} \cdot s^{-1}$, $6.0 \times 10^{-3}$ $s^{-1}$ and $2.75 \times 10^4$ $M^{-1} \cdot s^{-1}$ respectively in 50 mM-sodium phosphate buffer, pH 7.5, at 20 °C (Hermann *et al.*, 1983). As judged by c.d., the enzyme regains approx. 85% of its native secondary structure within 30 s of the start of the refolding process, showing that M in scheme (1) represents a folded monomer (Hermann *et al.*, 1983). M, D and T possess approx. 35%, 35% and 100% respectively of the activity of native enzyme; however, the activity of M and D differs from that of T in being sensitive to trypsin (Hermann *et al.*, 1985).

Susceptibility to proteolysis has proved to be a valuable method for studying structural changes in proteins during refolding (Girg *et al.*, 1981; Price & Stevens, 1983*a*; Schmid & Blaschek, 1984). In the present paper we have explored the sensitivity of the enzyme during refolding to three different proteinases, trypsin, chymotrypsin and thermolysin. In all three cases there is a substantial loss of activity in the early stages of refolding. Analysis by SDS/polyacrylamide-gel electrophoresis shows that this loss of activity is associated with extensive digestion of the polypeptide chain. There was no evidence for the production of substantial quantities of fragments of $M_r$ greater than 6500, suggesting that the early formed intermediates possess a number of sites that are susceptible to proteolysis.

## MATERIALS AND METHODS

The following companies supplied the reagents listed: BDH Chemicals, guanidinium chloride (AristaR grade); Sigma Chemical Co., Reactive Blue 2–Sepharose CL-6B, Dalton Mark VII-L $M_r$-marker set, turkey egg-white proteinase-inhibitor protein (type II-T), α-chymotrypsin-[tosyl-lysylchloromethane ('TLCK')-treated, dialysed, freeze-dried], subtilisin BPN', elastase (type IV) and thermolysin (stabilized with calcium acetate and sodium acetate); Boehringer Mannheim, proteinase K from *Tritirachium album* Limber, 3-phosphoglycerate (grade I), 2,3-bisphosphoglycerate and enolase; Worthington Corp., trypsin [three times recrystallized, tosylphenylalanylchloromethane('TPCK')-treated];Bayer,aprotinin (Trasylol).

Phosphoglycerate mutase was initially prepared from baker's yeast by the method reported previously (Price & Jaenicke, 1982). However, some difficulties were encountered in reproducing the ion-exchange step with different batches of DEAE-cellulose. An alternative method of purification was devised, taking advantage of the finding that yeast phosphoglycerate mutase binds to Cibacron Blue–Sepharose and can be eluted by bisphosphoglycerate (Price & Stevens, 1983*b*). The purification procedure of de la Morena *et al.* (1968) was followed as far as fraction 3, which was then dialysed overnight (at 4 °C) against 50 mM-sodium phosphate buffer, pH 7.5. The protein precipitated between 58% and 70% saturation with $(NH_4)_2SO_4$ was collected and re-extracted by the procedure of de la Morena *et al.* (1968), and the fraction containing maximal activity was dialysed against 10 mM-Tris/HCl buffer, pH 8.0. The dialysis residue was then applied to a column (10 cm × 1.8 $cm^2$) of Cibacron

Blue–Sepharose equilibrated against the Tris buffer. Unbound proteins were eluted by a wash with 20 ml of Tris buffer, and then some of the contaminating proteins (probably dehydrogenases and/or kinases) were eluted by a wash with 20 ml of Tris buffer containing 1 mM-AMP. Phosphoglycerate mutase was then eluted by a wash with 20 ml of Tris buffer containing 1 mM-bisphosphoglycerate. The fractions containing activity were combined, and the enzyme was precipitated by addition of solid $(NH_4)_2SO_4$ to 80% saturation. Analysis by SDS/polyacrylamide-gel electrophoresis showed that the enzyme was at least 95% homogeneous by staining with Coomassie Blue, and the specific activity (850 $\mu$mol of 3-phosphoglycerate consumed/min per mg of protein) was comparable with previously reported values. The assay of enzyme activity by an enolase-coupled assay and spectrophotometric determination of protein concentration were performed as described previously (Hermann *et al.*, 1983).

Turkey egg-white proteinase-inhibitor protein was coupled to Sepharose 4B by using the CNBr activation procedure (March *et al.*, 1974). The amount of inhibitor bound to the gel was determined as 2.0 mg/ml of packed gel by measuring the protein removed after reaction by a series of sequential washes at pH 9.5 and pH 4.0. Control experiments showed that the immobilized inhibitor protein inactivated at least 97% of the activity of chymotrypsin or trypsin in solution under the conditions used in the refolding experiments (see below).

Denaturation in guanidinium chloride and refolding of phosphoglycerate mutase were performed as described previously (Hermann *et al.*, 1983). Samples withdrawn from the renaturation mixture at various times were diluted if necessary to a standard protein concentration of 10 $\mu$g/ml, and incubated for 1 min at 20 °C with proteinase (20 $\mu$g/ml or 5 $\mu$g/ml) before enzyme assay or analysis by SDS/polyacrylamide-gel electrophoresis with the buffer system of Laemmli (1970) with 15%-acrylamide slab gels of 1.5 mm thickness. The inhibition of thermolysin before SDS/polyacrylamide-gel electrophoresis was performed by addition of EDTA (final concentration 2 mM) (Girg *et al.*, 1981). Trypsin and chymotrypsin could be effectively inhibited by addition of SDS to a final concentration of 0.1% followed by rapid transfer to a boiling-water bath and incubation for 2 min at 100 °C (Price & Stevens, 1982). Alternatively, the sample (3 ml) was mixed with 0.25 ml of immobilized turkey egg-white proteinase-inhibitor protein and centrifuged (1000 *g* for 1 min) before addition of SDS and boiling. The latter method had the advantage that trypsin and chymotrypsin could be effectively removed from solution, and hence bands due to these proteinases did not appear on SDS/polyacrylamide-gel electrophoresis. For the determination of $M_r$ the Dalton Mark VII-L marker set was supplemented by addition of aprotinin ($M_r$ 6500).

Staining and destaining of gels with Coomassie Blue was performed by the procedure of Laemmli (1970). The gels were scanned with a Gelman DCD 16 scanner. Subsequent silver staining of the gels was performed by the method of Wray *et al.* (1981).

## RESULTS AND DISCUSSION

### Regain of activity at different enzyme concentrations

As shown in Fig. 1, phosphoglycerate mutase activity was regained rapidly following the dilution of guanidinium chloride to 0.1 M. Data are shown for final enzyme concentrations of 10, 30 and 50 $\mu$g/ml; the continuous lines in the Figure are calculated on the basis of the model (scheme 1) in which, in the absence of proteinases, the monomeric and dimeric species possess 35% of the activity of the tetramer (Hermann *et al.*, 1985).

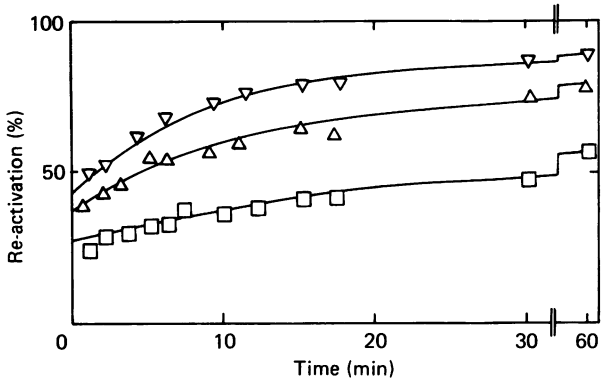


**Fig. 1. Re-activation of phosphoglycerate mutase**

Enzyme was denatured by incubation in 4 M-guanidinium chloride and renatured by dilution of the guanidinium chloride to 0.1 M in 50 mM-sodium phosphate buffer, pH 7.5, at 20 °C. The activity of samples taken was assayed in the absence of added proteinase. Final concentration during renaturation: ▽, 50 $\mu$g/ml; △, 30 $\mu$g/ml; □, 10 $\mu$g/ml. Continuous lines were calculated assuming the model shown in scheme (1), with the monomeric and dimeric intermediates each possessing 35% of the activity of the tetramer (Hermann *et al.*, 1985).

### Susceptibility of native enzyme to proteolysis

In order to decide which proteinases might be suitable for exploring structural changes in the enzyme during refolding, some experiments were carried out in which native enzyme (400 $\mu$g/ml) was incubated with various proteinases in 50 mM-sodium phosphate buffer, pH 7.5, at 20 °C: trypsin (40 $\mu$g/ml), chymotrypsin (40 $\mu$g/ml), thermolysin (40 $\mu$g/ml), elastase (20 $\mu$g/ml), subtilisin (10 $\mu$g/ml) and proteinase K (10 $\mu$g/ml). Under these conditions native enzyme was relatively stable towards trypsin, chymotrypsin and thermolysin ($\leqslant$ 10% loss of activity after 5 min, 40–65% loss after 60 min) but much less stable towards elastase, subtilisin and proteinase K (50%, 65% and 65% loss of activity respectively after 5 min). In general, these rates of loss of activity were correlated with the rates of loss of the intact subunit polypeptide chain ($M_r$ 28000), as detected by SDS/polyacrylamide-gel electrophoresis. Since intermediates of refolding are likely to be more susceptible than native enzyme to proteolysis (Girg *et al.*, 1981), it was concluded that, of the proteinases tested, trypsin, chymotrypsin and thermolysin afforded the best possibilities of exploring structural changes in the enzyme during refolding.

### Effect of proteinases on enzyme activity during refolding

When the activities of phosphoglycerate mutase were assayed after 1 min incubation with proteinase, there was a marked decrease in activity regained, compared with control samples that had been incubated for 1 min in the absence of proteinase before assay; this decrease was especially marked at the early time points. Fig. 2 shows the results obtained at a phosphoglycerate mutase

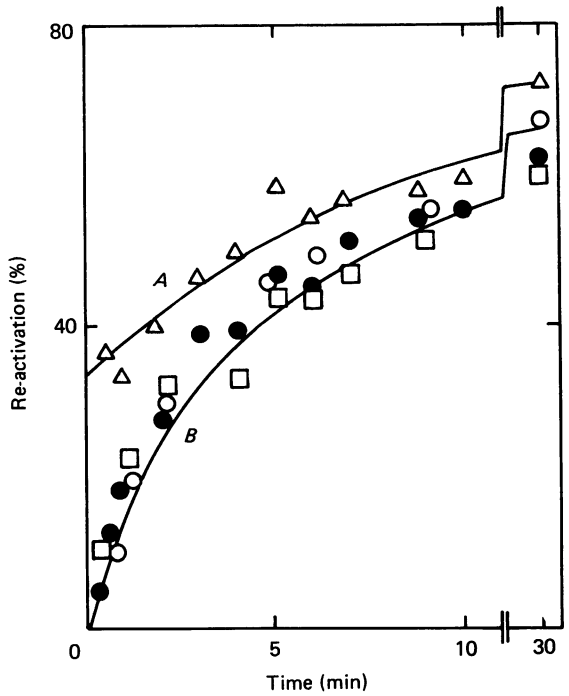


**Fig. 2. Effect of proteinases on the re-activation of phosphoglycerate mutase**

The final concentration of phosphoglycerate mutase during renaturation was 30 $\mu$g/ml. △, Activity assayed in the absence of added proteinase; ●, ○ and □, activity assayed after 1 min incubation with trypsin, chymotrypsin and thermolysin respectively (each 20 $\mu$g/ml). The results obtained after incubation with proteinases at 5 $\mu$g/ml were qualitatively similar although the effects were rather less marked. Other conditions were as indicated in Fig. 1 legend. The continuous line *A* was calculated by using the model of scheme (1), with the monomeric and dimeric intermediates each assumed to possess 35% of the activity of the tetramer. The continuous line *B* was calculated by assuming these intermediates to possess zero activity.

concentration of 30 $\mu$g/ml during refolding; the results at 10 $\mu$g/ml and 50 $\mu$g/ml showed a similar effect. Under the conditions chosen, the activity of native phosphoglycerate mutase (in the absence or in the presence of 0.1 M-guanidinium chloride, the residual concentration of denaturant during refolding) was not significantly affected by incubation with the proteinases for 1 min. The continuous line *A* in Fig. 2 is calculated on the basis of the line in Fig. 1, i.e. that monomeric and dimeric species each possess 35% of the activity of the tetramer. The continuous line *B* in Fig. 2 is calculated on the basis that the monomeric and dimeric species possess zero activity. The results obtained in the presence of the proteinases show reasonable agreement with the calculated line *B*, thus confirming the earlier conclusion (Hermann *et al.*, 1985) that the activities of the monomeric and dimeric species are sensitive to trypsin, and extending this conclusion to the effects of other proteinases with differing bond specificities.

### Digestion of phosphoglycerate mutase by proteinases during refolding

SDS/polyacrylamide-gel electrophoresis was used to examine the digestion by proteinases of the enzyme

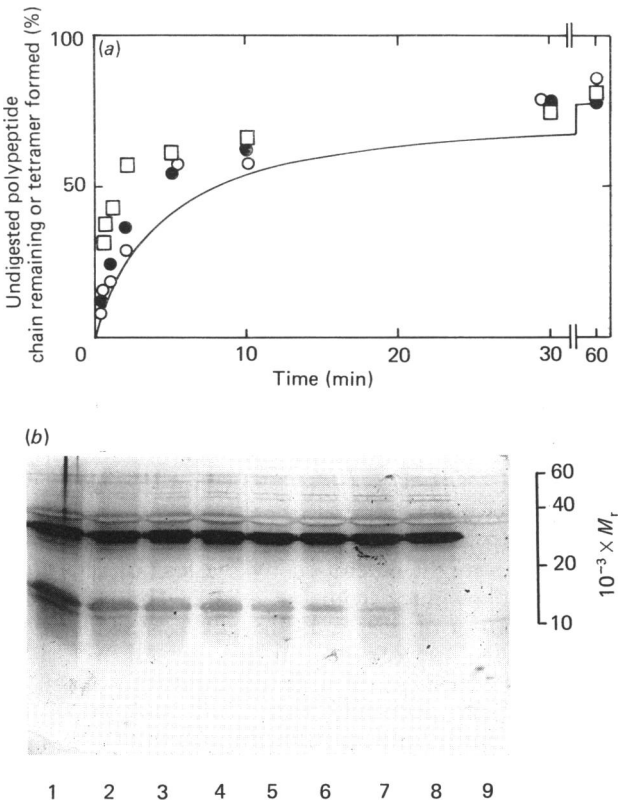


**Fig. 3. Digestion of phosphoglycerate mutase by proteinases during refolding**

Conditions were as indicated in Fig. 2 legend. Samples taken during renaturation were incubated for 1 min with proteinase (20 $\mu$g/ml) and then analysed by SDS/polyacrylamide-gel electrophoresis. (*a*) Percentage of undigested polypeptide chain ($M_r$ 28000) remaining plotted as a function of time of renaturation. ●, ○ and □ represent digestion by trypsin, chymotrypsin and thermolysin respectively. The continuous line is the percentage of tetramer formed, calculated in accordance with scheme (1) (see line *B* in Fig. 2). (*b*) SDS/polyacrylamide gel electrophoresis of samples incubated with thermolysin. The gel was stained with silver according to the method of Wray *et al.* (1981). Lanes 1–7 represent samples taken after 0.25, 0.5, 1, 2, 5, 30 and 60 min renaturation respectively. Lane 8 represents native phosphoglycerate mutase incubated with thermolysin under these conditions. Lane 9 represents thermolysin. The $M_r$ scale from standard proteins is shown on the right. Possible reasons for apparent negative silver staining of some proteins (e.g. thermolysin) have been discussed by Morrissey (1981).

during refolding. The results of these experiments are shown in Fig. 3(*a*) for the refolding experiments performed at a phosphoglycerate mutase concentration of 30 $\mu$g/ml and subsequent incubation with 20 $\mu$g of proteinase/ml for 1 min. The ordinate represents the percentage of intact polypeptide chain ($M_r$ 28000) material remaining after incubation with the proteinase relative to the control sample, which represents native enzyme. (It should be noted that incubation of native enzyme with the proteinases under these conditions caused no significant digestion of the polypeptide chain.) It is clear that for all three proteinases tested (trypsin, chymotrypsin and thermolysin) there is very considerable

digestion of the polypeptide chain at the early times of refolding and relatively little digestion at the later times. In general, and especially for thermolysin, the percentage of 28000-$M_r$ material remaining runs somewhat ahead of the percentage of tetramer formed (shown as the continuous line in Fig. 3*a*), which implies that the monomeric and dimeric species are not completely digested under these conditions. However, by increasing the concentration of proteinase, a greater degree of digestion can be obtained. Thus, by using 80 $\mu$g of thermolysin/ml, the amount of 28000-$M_r$ material remaining in the sample taken at 0.25 min is reduced to 20%, compared with 32% at 20 $\mu$g of thermolysin/ml. (This higher concentration of thermolysin did not cause any digestion of native enzyme under these conditions.)

It is not clear why the degree of inactivation caused by proteinases (Fig. 2) does not correlate exactly with the loss of the 28000-$M_r$ band shown in Fig. 3(*a*). The discrepancy could reflect the greater degree of experimental error in the latter measurements or indicate that inactivation might be caused by the loss of only a few amino acid residues from one end of the polypeptide chain (Winn *et al.*, 1981; Fothergill & Harkins, 1982). Some of the inactivated enzyme, which would not necessarily be resolved from native enzyme by SDS/polyacrylamide-gel electrophoresis under our conditions, could also undergo more extensive digestion.

The gels were examined carefully for evidence of distinct fragments formed by the action of proteinases on samples during refolding. Such fragments would presumably represent compact folded units, which would be resistant to digestion by proteinases. In the cases of trypsin and chymotrypsin, there was no evidence for any distinct fragments of $M_r$ greater than 6500 in any samples during refolding (from 0.25 min to 60 min). This conclusion was reached with the use of Coomassie Blue or the more sensitive silver staining procedure. In the case of thermolysin, staining with Coomassie Blue (and more particularly with silver) revealed the presence of fragments in the 12000–15000-$M_r$ range from samples taken at early time points but to a lesser extent from those taken from later time points (Fig. 3*b*). However, the degree of staining by Coomassie Blue of these fragments was in all cases at least an order of magnitude less than the 28000-$M_r$ band of the control sample, showing that even at the early time points most of the polypeptide chain had been degraded to small fragments ($M_r$ less than 6500).

The overall conclusion from these experiments is that, at early time points (less than 2 min) during refolding, when the folded monomeric form predominates (Hermann *et al.*, 1983, 1985), the polypeptide chain is highly susceptible to the action of the proteinases trypsin, chymotrypsin and thermolysin. Since most of the enzyme appears to be digested to fragments of $M_r$ less than 6500, it would appear that the folded monomeric, and probably the dimeric, species have a large number of susceptible bonds. It is noteworthy that the c.d. experiments (Hermann *et al.*, 1983) show that the folded monomer possesses approx. 85% of the secondary structure of native enzyme. The results in the present paper imply that this folded structure is still rather 'loose', with a number of exposed sites for attack by proteinases; these sites are presumably rendered less accessible during subsequent association of the monomers (and dimers) and/or 'tightening' of the subunit structure. The small amount of fragments generated by thermolysin at short times of refolding (Fig. 3*b*) implies that at least one of the first bonds to be cleaved in the folded monomer is located near the centre of the polypeptide chain. Examination of the amino acid sequence of the enzyme (Fothergill & Harkins, 1982) reveals a tetraproline sequence (119–122) almost exactly in the centre of the chain of 241 amino acids. Since the initial events in the folding of polypeptide chains are thought to involve formation of secondary structures such as $\alpha$-helices (Jaenicke, 1982) that cannot accommodate proline residues (Chou & Fasman, 1978), it is tempting to postulate that an early event in the refolding of the phosphoglycerate mutase subunit involves the separate folding of the two halves of the polypeptide chain, so as to leave exposed one or more bonds near the tetraproline sequence. The purification and further characterization of proteolytic fragments will be necessary to provide confirmation of this hypothesis.

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## REFERENCES

Chan, W. W.-C., Mort, J. S., Chong, D. K. K. & Macdonald, P. D. M. (1973) J. Biol. Chem. **248**, 2778–2784
Chou, P. Y. & Fasman, G. D. (1978) Annu. Rev. Biochem. **47**, 251–276
de la Morena, E., Santos, I. & Grisolia, S. (1968) Biochim. Biophys. Acta **151**, 526–528
Fothergill, L. A. & Harkins, R. N. (1982) Proc. R. Soc. London Ser. B **215**, 19–44
Girg, R., Rudolph, R. & Jaenicke, R. (1981) Eur. J. Biochem. **119**, 301–305
Grossman, S. H., Pyle, J. & Steiner, R.J. (1981) Biochemistry **20**, 6122–6128
Hermann, R., Jaenicke, R. & Rudolph, R. (1981) Biochemistry **20**, 5195–5201
Hermann, R., Rudolph, R., Jaenicke, R., Price, N. C. & Scobbie, A. (1983) J. Biol. Chem. **258**, 11014–11019
Hermann, R., Jaenicke, R. & Price, N. C. (1985) Biochemistry **24**, 1817–1821
Jaenicke, R. (1982) Biophys. Struct. Mech. **8**, 231–256
Jaenicke, R. (1984) Angew. Chem. Int. Ed. Engl. **23**, 395–413
Jaenicke, R. & Rudolph, R. (1983) in Biological Oxidations (Sund, H. & Ullrich, V., eds.), pp. 62–90, Springer-Verlag, Berlin and Heidelberg
Laemmli, U. K. (1970) Nature (London) **227**, 680–685
March, S. C., Parikh, I. & Cuatrecasas, P. (1974) Anal. Biochem. **60**, 149–152
Morrissey, J. H. (1981) Anal. Biochem. **117**, 307–310
Price, N. C. & Jaenicke, R. (1982) FEBS Lett. **143**, 283–286
Price, N. C. & Stevens, E. (1982) Biochem. J. **201**, 171–177
Price, N. C. & Stevens, E. (1983*a*) Biochem. J. **209**, 763–770
Price, N. C. & Stevens, E. (1983*b*) Biosci. Rep. **3**, 857–861
Rudolph, R., Westhof, E. & Jaenicke, R. (1977) FEBS Lett. **73**, 204–206
Schmid, F. X. & Blaschek, H. (1984) Biochemistry **23**, 2128–2133
Winn, S. I., Watson, H. C., Harkins, R. N. & Fothergill, L. A. (1981) Philos. Trans. R. Soc. London Ser. B **293**, 121–130
Wray, W., Boulikas, T., Wray, V. P. & Hancock, R. (1981) Anal. Biochem. **118**, 197–203

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