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Radiochemical Determination of a Unique Sequence around the Reactive Serine Residue of a Di-isopropyl Phosphorofluoridate-Sensitive Plant Carboxypeptidase and a Yeast Peptidase

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Phaseolain, a carboxypeptidase from French-bean leaves, and a partially purified peptidase from baker's yeast are inhibited by reaction with di-isopropyl phosphorofluoridate. Radioactive di-isopropyl [^{32}P]phosphorofluoridate was used to show that the site of reaction is a unique serine residue and that the sequence of amino acids adjacent to the reactive serine is Glu-Ser-Tyr. This sequence is different from those of other 'serine' enzymes previously reported and, for phaseolain, represents an unequivocal example of a 'serine' carboxypeptidase.

We reported (Shaw & Wells, 1967) that partially purified preparations of phaseolain, a carboxypeptidase from French beans, and a peptidase with similar properties from baker's yeast were inhibited by Dip-F*. The electrophoretic patterns of phosphoryl peptides derived from partial acid hydrolysates of the [^{32}P]Dip-F-labelled enzymes were identical and indicated that they contained a reactive serine residue in a sequence that was different from those in other 'serine' enzymes previously examined (Sanger, 1963).

The inter-relationships of the [^{32}P]phosphoryl peptides from the yeast peptidase were established radiochemically by subtractive Edman degradation, rehydrolysis and carboxypeptidase A digestion of the peptides. Details of this work are reported in the present paper together with the determination of the sequence around the reactive serine residue, which was established as Glu-Ser-Tyr.

Preparations of phaseolain completely free of aminopeptidase or endopeptidase contamination have been obtained (Carey & Wells, 1970). It is clear from examination of the phosphoryl peptides derived from such preparations treated with [^{32}P]-Dip-F that this purified plant carboxypeptidase contains a reactive serine residue within the sequence Glu-Ser-Tyr.

Materials and Methods

[^{32}P]Dip-F (200μCi/mg) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

* Abbreviations: Dip-F, di-isopropyl phosphorofluoridate; Dip, di-isopropoxyphosphinyl; Z, benzyloxycarbonyl; Ser(P), *O*-phosphoserine.

Preparation of phaseolain

The plant enzyme was purified as described previously (Wells, 1968; Carey & Wells, 1970).

Preparation of yeast peptidase

Extraction of enzyme. Fresh baker's yeast was crumbled and air-dried for 3 days at room temperature and was then ball-milled for 26 h at −15°C. The dry, white powder was extracted with 0.1 M-sodium acetate, pH 5.0 (1 litre of buffer/200 g of powder), for 16 h at 4°C. Insoluble material was removed by centrifugation (30000*g*, 30 min) and the supernatant was dialysed against 0.1 M-sodium acetate, pH 6.0 (16 h, 4°C). The precipitate that formed during dialysis was removed by centrifugation (20000*g*, 30 min).

DEAE-cellulose chromatography. This was done in two stages. (i) Small columns (8 cm × 1 cm) were poured with 5 g 'moist' weight of exchanger and equilibrated with 0.1 M-sodium acetate, pH 6.0. Portions (50 ml) of the above supernatant were pumped through each column at 30 ml/h, the columns were washed with a further 50 ml of equilibrating buffer and the enzyme was then eluted with the same buffer containing 1 M-NaCl. The first 5 ml portion of eluate from each column was discarded and the next 15 ml, containing the enzyme, was kept. (ii) The pooled 1 M-NaCl eluates (230 ml) were dialysed against 0.05 M-sodium acetate, pH 6 (16 h, 4°C), and chromatographed on a column of DEAE-cellulose (2.5 cm × 30 cm) equilibrated with the dialysing buffer. A convex gradient was generated with 200 ml portions of 0.05 M-sodium acetate, pH 6, containing respectively 0.1 M-, 0.5 M- and 1 M-NaCl in

three interconnected Autograd chambers (Technicon Instruments Corp., Chauncey, N.Y., U.S.A.). Two peaks with activity towards Z-L-Phe-L-Leu were eluted; the most active fractions of the first peak, which contained most of the total peptidase activity (40ml), were concentrated to 1ml by ultrafiltration.

Sephadex chromatography. The enzyme sample was chromatographed on a column (2.5cm×40cm) of Sephadex G-200 equilibrated with 0.05M-sodium acetate, pH6.0. The elution volume of this yeast peptidase was similar to that of the plant enzyme, phaseolain, for which a molecular weight of 120000 has been estimated (Wells, 1968). The active fractions were combined (45ml). The preparation contained 0.82mg of protein/ml and had a specific activity (μmol of leucine hydrolysed/min per mg of protein) of 25.6 towards Z-L-Phe-L-Leu.

Peptidase assay

The yeast enzyme and phaseolain were assayed by measurement of leucine released from the substrate Z-L-Phe-L-Leu (Wells, 1965). Inhibition of activity during incubation with [^{32}P]Dip-F was detected by a semi-quantitative assay. A portion (20μl) of the treated enzyme was removed, mixed with 50μl of substrate (0.002M-Z-L-Phe-L-Leu in 0.05M-sodium acetate, pH6) and 10μl samples were spotted and dried on Whatman 3MM paper immediately and after a further incubation time of 20min. Enzymic activity (or the lack of it) was readily observed after dipping the paper strips in ninhydrin (0.1% in acetone) to detect the leucine released.

Labelling of enzymes

Portions (5–10ml) of enzyme solution were treated with [^{32}P]Dip-F (0.4ml, 0.02M in propan-2-ol). After incubation (room temperature, 4–8h) the mixture was dialysed for 2 days against three changes of 5 litres of 0.01M-acetic acid, and then freeze-dried.

Investigation of [^{32}P]phosphoryl peptides

The radiochemical techniques and methods used have been described by Naughton *et al.* (1960) and more recently applied by Larner & Sanger (1965). Partial acid hydrolysis was performed in 6M-HCl at 100°C for 30min. Subtractive Edman degradation was done as follows. The radioactive peptide was dissolved in 0.1ml of water. Phenyl isothiocyanate in pyridine (0.2ml; 1:19, v/v) was added. This mixture should be a one-phase system; if not, pyridine was added dropwise until this was achieved, and the mixture was then left at room temperature for 4–6h. Benzene and a few drops of water were added and the mixture was shaken vigorously, then the benzene layer was removed. The extraction was repeated until the aqueous layer was crystal clear; the aqueous layer was evaporated to dryness over H_2SO_4 in a desiccator. After addition of water (0.2ml) and evaporation twice, 0.2ml of acetic acid–6M-HCl (1:1, v/v) was added and the mixture was left for 4–6h. The acid was removed *in vacuo* over NaOH and after further addition of water and redrying, the sample was ready for electrophoresis in parallel with a marker hydrolysate, to identify the residual radioactive peptide. Rehydrolysis indicates hydrolysis by 6M-HCl at 100°C for 30min unless otherwise specified. Carboxypeptidase A hydrolysis was performed in aq. 0.5% (w/v) NH_4HCO_3 at 37°C for 5h. Carboxypeptidase A was a Dip-F-treated preparation from Worthington Biochemical Corp., Freehold, N.J., U.S.A.

Marker peptides labelled with ^{32}P. These were obtained from partial acid hydrolysates of [^{32}P]Dip-F-treated chymotrypsin and subtilopeptidase A by published procedures (Naughton *et al.*, 1960; Sanger & Shaw, 1960). A useful reference pattern was also obtained by treating human milk with [^{32}P]Dip-F. Freshly collected milk (5ml) was centrifuged at 10000*g* for 10min. Sodium phosphate buffer (1ml, 0.5M, pH7.6) was then added to the clear whey and the solution was treated with [^{32}P]Dip-F. It had been found (Shaw, 1962) that a partial acid hydrolysate of the dialysed freeze-dried treated milk gave a clean pattern of inter-related phosphoryl peptides corresponding to the sequence Glu-Ser(P)-Ala-Gly, presumably owing to labelling of an esterase present in the human milk. This preparation is referred to as 'milk enzyme'.

Synthetic dipeptides containing O-phosphoserine. Ser(P)-Val was prepared by direct phosphorylation of Ser-Val (Cyclo Chemical Corp., Los Angeles, Calif., U.S.A.) with phosphorus oxychloride (Neuhaus & Korkes, 1958). Leu-Ser(P), (kindly provided by Dr. C. Milstein, M.R.C. Laboratory of Molecular Biology, Cambridge, U.K.) was heated in 0.1M-HCl to give the inversion product Ser(P)-Leu (Shaw, 1962). A mixutre of Ser(P)-Ala and Ser(P)-Ile was obtained from the eluate of a partial acid hydrolysate of ovalbumin applied to a column of Dowex 50 (H^+ form) (Shaw, 1962). Ser(P)-Tyr was prepared on a small scale by the coupling of *O*-phosphoserine and tyrosine benzyl ester (Hoare & Koshland, 1967) in the presence of 1-cyclohexyl-3-(2-morpholinoethyl)carbodi-imide methyl-*p*-toluene sulphonate (Aldrich Chemical Co., Milwaukee, Wis., U.S.A.) followed by hydrolysis of the benzyl ester with 6M-HCl for 30min at 100°C. Electrophoresis at pH3.5 of the hydrolysed mixture showed four ninhydrin-positive compounds, two of which were unchanged *O*-phosphoserine and tyrosine. Another band (later shown to have the same electrophoretic mobility as radioactive band 3; see Fig. 2) yielded equimolar amounts of serine and tyrosine

after total acid hydrolysis, and Edman degradation of this material gave free tyrosine as the product. The fourth band (of slightly greater mobility) also yielded equimolar amounts of serine and tyrosine after acid hydrolysis and was probably the pyrophosphate derivative of the required dipeptide.

Chromatography of amino acids and peptides

Three solvent systems were used. These were: A, butan-1-ol – acetic acid – water – pyridine (15:3:12:10, by vol.) (Waley & Watson, 1953); B, pyridine–2-methylbutan-2-ol–water (7:7:6, by vol.) (Moore & Baker, 1958); C, phenol saturated with aq. 10% (w/v) trisodium citrate (Moore & Baker, 1958).

Results and Discussion

Peptidase inhibition by [^{32}P]Dip-F

Samples of the enzyme solution were assayed on paper strips for peptidase activity during incubation with [^{32}P]Dip-F; at least 90% of the activity was lost within 30min of the addition of the inhibitor. It had been shown (J. R. E. Wells, unpublished work) that the plant enzyme phaseolain and a preparation of yeast peptidase purified as described in the Materials and Methods section were completely inhibited by preincubation with unlabelled Dip-F.

Pattern of phosphoryl peptides from [^{32}P]Dip-yeast peptidase hydrolysate

The pattern of phosphoryl peptides seen after electrophoretic separation at pH3.5 of the partial acid hydrolysate of the [^{32}P]Dip-F-treated yeast enzyme is clearly different from those obtained from hydrolysates of Dip-F-treated chymotrypsin, subtilopeptidase A and milk enzyme, which were run in parallel (Plate 1). P_i and *O*-phosphoserine are common to all four hydrolysates, but the patterns of labelled peptide bands are different and indicate different sequences about the reactive serine residue in each case. A strip containing the peptide bands derived from the yeast enzyme was cut from the pH3.5 electrophoretogram and subjected to electrophoresis at right angles at pH6.5. A tracing of this two-dimensional separation is shown in Fig. 1 on which the peptides have been numbered. There are five major phosphoryl peptides, 1, 3a, 3, 4 and 5. The peptides 1, 3a and 4 show an apparent increase in net negative charge at pH6.5 compared with bands 3 and 5, the former group moving relatively faster than they did at pH3.5.

Samples of each of the bands were eluted and subjected to a further acid hydrolysis for 30min, Edman degradation or incubation with carboxypeptidase A. The radioactive products obtained from each were characterized by electrophoresis and are listed in Table 1. The results indicate that the peptide bands are inter-related within a sequence A-Ser(P)-X-Y (where A, X and Y represent amino acids) the probable structure of each peptide being shown in Table 1.

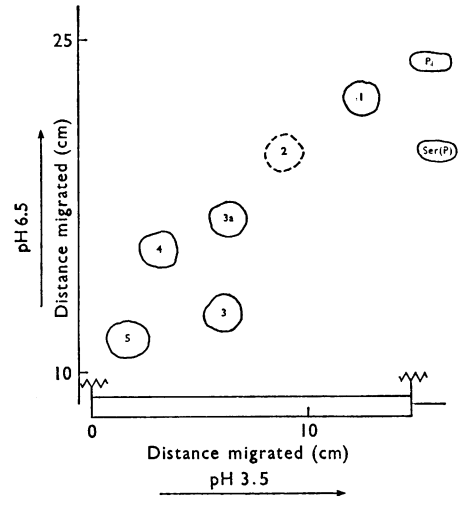


Fig. 1. *Tracing of radioautograph of two-dimensional separation of phosphoryl peptides from a partial acid hydrolysate of Dip-yeast peptidase*

The first dimension was in pyridine–acetate buffer, pH3.5 [aq. 0.5% (v/v) pyridine, 5% (v/v) acetic acid], at 40V/cm for 2h and the second dimension was in pyridine–acetate buffer, pH6.5 [aq. 10% (v/v) pyridine, 0.5% (v/v) acetic acid], at 40V/cm for 1h. The numbering of bands used in the text is indicated. Markers of P_i and Ser(P) were subjected to electrophoresis in the second dimension only.

Investigation of peptide 1. Band 1 on subtractive Edman degradation gave Ser(P) and so must be a dipeptide A-Ser(P); the products of rehydrolysis were also consistent with this conclusion. The band had the same mobility at pH3.5 (Plate 1) as a band in the [^{32}P]Dip-milk enzyme hydrolysate, known to be Glu-Ser(P) (Shaw, 1962). Comparison of mobility of the peptides from the two sources was extended to electrophoresis at pH4.7 and 6.5 and chromatography in systems A, B and C. The two had identical mobilities in all systems. Above pH3.5 Glu-Ser(P) gains a full extra negative charge and moves relatively faster than a dipeptide involving *O*-phosphoserine and a neutral amino acid. This increase in charge has been noted also for bands 3a and 4 (Table 1) and would indicate that each of these peptides contains this acidic residue A within its sequence.

Table 1. *Examination of bands from* [^{32}P]*Dip-yeast peptidase hydrolysate*

For experimental details see the text.

Band no.	Products of: Rehydrolysis	Edman degradation	Carboxypeptidase hydrolysis	Extra negative charge at pH 6.5	Probable structure
1	1	Ser(P)	1	Yes	A-Ser(P)
3	3	P_i	3	No	Ser(P)-X
3a	1, 3	3	3a, 1	Yes	A-Ser(P)-X
4	1, 3, 3a, 5	5	3a, 1	Yes	A-Ser(P)-X-Y
5	3	P_i	3	No	SerP-(X)-Y

Investigation of peptides 3 *and* 3a. Peptides 3 and 3a ran together on electrophoresis at pH3.5, but separated at higher pH values, as peptide 3a gained an extra negative charge. Peptide 3 was essentially stable to rehydrolysis (30min) with acid and yielded only traces of *O*-phosphoserine and P_i as radioactive products. Edman degradation of peptide 3 yielded only P_i, which has been observed for peptides with *N*-terminal *O*-phosphoserine (Naughton *et al.*, 1960). In the time-course of acid hydrolysis (see Naughton *et al.*, 1960) an extra band just ahead of band 3 was observed with longer times of hydrolysis (about 3h). This extra band was also observed when peptide 3 was heated in 0.1M-HCl at 100°C for 6h in an evacuated sealed tube, if phenol had been added to the hydrolysate (as discussed below). These general conditions have been used for the inversion of dipeptide sequences (Larner & Sanger, 1965; Shaw, 1962). The extra band, although faster-moving than peptide 3 at pH3.5, was slower-moving at pH6.5, a behaviour previously noted for the inverted forms of *O*-phosphoserinyl dipeptides (Shaw, 1962). Subtractive Edman degradation of the extra band yielded *O*-phosphoserine, strongly suggesting that the extra band was the inverted form [X-Ser(P)] of peptide 3, and more particularly that band 3 represented the dipeptide Ser(P)-X.

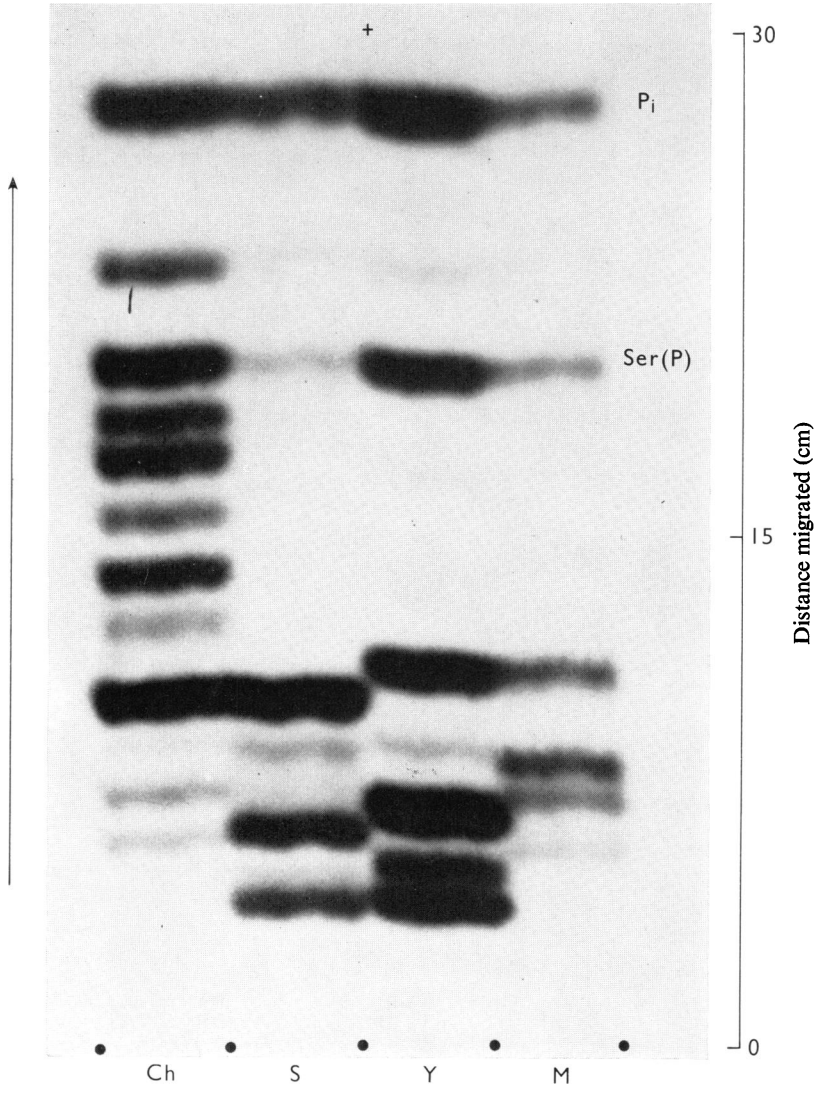
Peptide 3a was separated from peptide 3 by electrophoresis at pH6.5. Subtractive Edman degradation of peptide 3a gave rise to peptide 3 (as judged by electrophoresis at pH3.5 and 6.5), and rehydrolysis of peptide 3a with acid gave peptides 1 and 3. Incubation of peptide 3a with carboxypeptidase A yielded some of band 1. All of these results are consistent with band 3a representing the tripeptide Glu-Ser(P)-X.

Investigation of peptides 4 *and* 5. Peptide 4 on rehydrolysis gave rise to bands 1, 3, 3a and 5, and subtractive Edman degradation of peptide 4 gave rise to band 5. Edman degradation of band 5 produced P_i, indicating that *O*-phosphoserine had been *N*-terminal, and rehydrolysis of peptide 5 gave the dipeptide 3. Incubation of peptide 4 with carboxypeptidase A yielded bands 3a and 1, and the same treatment of peptide 5 gave rise to the dipeptide 3. Peptide 5 therefore appears to be the tripeptide, Ser(P)-X-Y, and the simplest interpretation of band 4 is that it is the glutamyl tetrapeptide, Glu-Ser(P)-X-Y.

Characterization of residue X

Electrophoretic and chromatographic studies. To identify the amino acid X, it was necessary to examine the mobility and behaviour of the radioactive dipeptide, Ser(P)-X, or larger peptides containing X. In the preliminary investigation (Shaw & Wells, 1967) it was noted that at pH3.5 the dipeptide Ser(P)-X (band 3) had a mobility intermediate between that of the markers Ser(P)-Ala and Ser(P)-Ile. These dipeptides had been encountered in Dip-F-treated esterase and in one of the phosphoserine sequences in ovalbumin respectively (Shaw, 1962). Although the mobility of peptides at pH3.5 will depend upon the weight of the peptide and the pK_a of carboxyl groups, it was expected that Ser(P)-Val would have a mobility very close to that of Ser(P)-X, and Ser(P)-Leu was expected to have the same mobility as Ser(P)-Ile. The removal of residue X from the tripeptide 3a by carboxypeptidase A to yield Glu-Ser(P) suggested that residue X was unlikely to be proline (considering the specificity requirements of carboxypeptidase A), although Ser(P)-Pro has the same molecular weight as Ser(P)-Val.

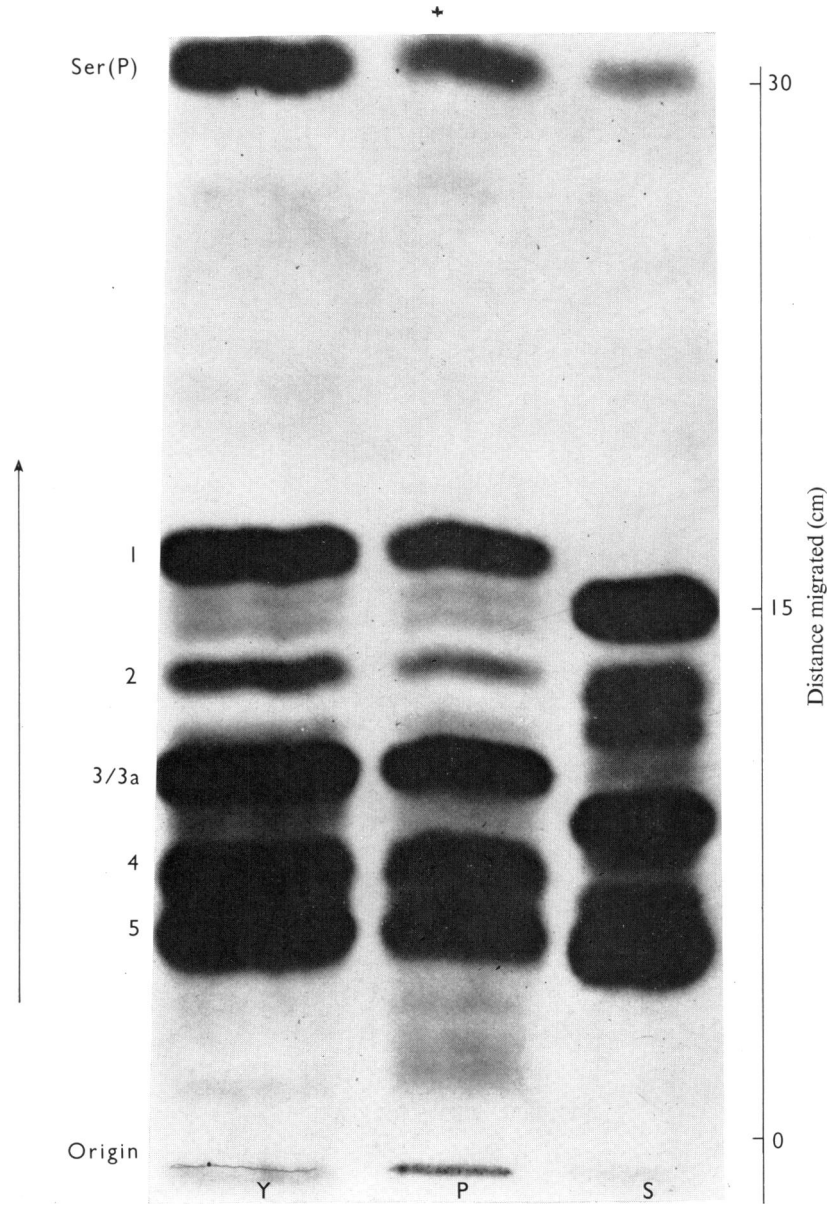
We have made a direct comparison of the electrophoretic mobilities at various pH values of dipeptides containing Ser(P) (Fig. 2). The markers Ser(P)-Ala, Ser(P)-Val, Ser(P)-Leu and Ser(P)-Ile were subjected to electrophoresis alongside band 3 at pH3.5, 4.7, 8.9 and 12. The results at pH8.9 were essentially the same as at pH4.7 and only the latter are shown. The markers were detected with ninhydrin and band 3



EXPLANATION OF PLATE 1

Radioautograph of partial acid hydrolysates of [^{32}*P*]*Dip-F-treated enzymes*

The hydrolysates were subjected to electrophoresis in pyridine–acetate buffer, pH 3.5 [aq. 0.5% (v/v) pyridine, 5% (v/v) acetic acid], at 40 V/cm for 1.5 h. Abbreviations: Dip-chymotrypsin (Ch), Dip-subtilopeptidase A (S), Dip-yeast peptidase (Y), and Dip-milk enzyme (M). The positions of inorganic phosphate (P_i) and Ser(P), which are common to all hydrolysates, are indicated.



EXPLANATION OF PLATE 2

Radioautograph of partial acid hydrolysates of [^{32}P]*Dip-F-treated yeast peptidase* (*Y*), *phaseolain* (*P*) *and subtilopeptidase A* (*S*)

Samples were subjected to electrophoresis in pyridine–acetate buffer, pH3.5 [aq. 0.5% (v/v) pyridine, 5% (v/v) acetic acid], at 40V/cm for 2h.

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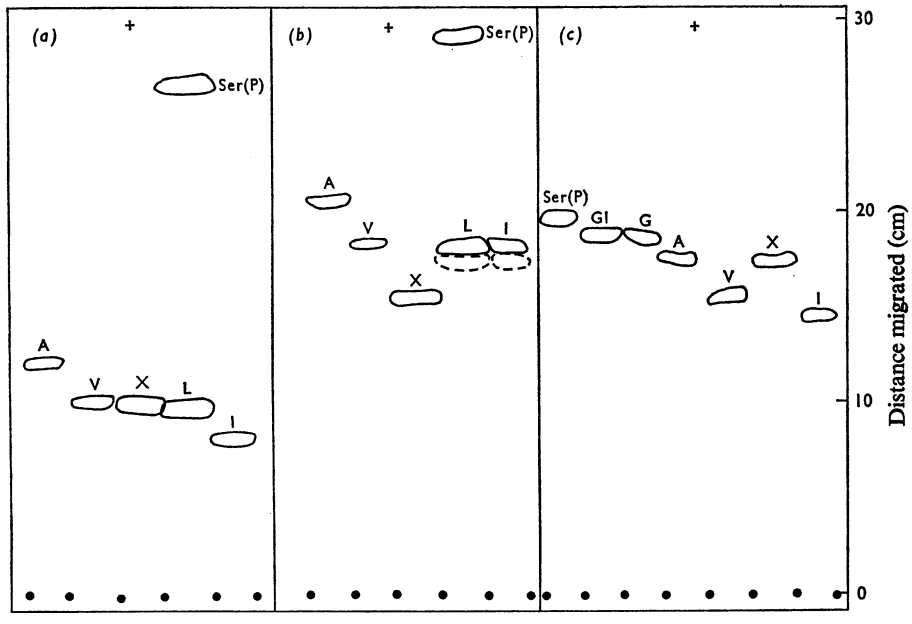

Fig. 2. *Tracing of electrophoretic separation of dipeptides containing Ser(P) at (a) pH3.5, (b) pH4.7 and (c) pH12*

For experimental details see the text. Pyridine–acetate buffers were used at (*a*) pH3.5 [aq. 0.5% (v/v) pyridine, 5% (v/v) acetic acid], (*b*) pH4.7 [aq. 2.5% (v/v) pyridine, 2.5% (v/v) acetic acid] and (*c*) 0.1M-Na_3PO_4 at pH12. Electrophoresis was at 40V/cm for (*a*) 2h, (*b*) 1.8h or (*c*) 0.5h. Abbreviations: G, Ser(P)-Gly; A, Ser(P)-Ala; V, Ser(P)-Val; X, Ser(P)-X (peptide 3); L, Ser(P)-Leu; I, Ser(P)-Ile. In (*c*) Gl is the peptide Ser(P)-Glu; in (*b*) ---- represents the inverted dipeptides Leu-Ser(P) and Ile-Ser(P) respectively. Marker dipeptides were located with ninhydrin and peptide 3 was located by radioautography.

was detected by radioautography. Fig 2 shows that at pH3.5, peptide 3 was slightly slower in moving towards the anode than Ser(P)-Val, and Ser(P)-Leu was faster-moving than Ser(P)-Ile and close to Ser(P)-X and Ser(P)-Val. Extending the comparison of mobilities to pH4.7 (Fig. 2) it was found that peptide 3 was appreciably slower-moving than any of the other *O*-phosphoseryl dipeptides. Residue X is obviously not valine or leucine, but behaves at pH4.7 (and pH8.9) as though it is heavier than either of these. The relatively greater mobility at pH3.5 of peptide 3 compared with the dipeptides containing aliphatic residues suggests that the pK_a of the carboxyl group in the dipeptide Ser(P)-X must be lower than for the aliphatic series. This greater acidity would then contribute to the relatively greater mobility of Ser(P)-X at pH3.5, whereas at higher pH values the carboxyl group of all the peptides would be fully ionized.

At this stage all the information suggested that residue X carried no net charge, but was not an aliphatic amino acid. The isolation of peptides 3, 4 and 5 in good yield argued against residue X being either threonine or serine, as partial acid hydrolysis of proteins results in preferential cleavage on the *N*-terminal side of such residues (Desnuelle & Casal, 1948). Direct comparison of dipeptide 3 with Ser(P)-Met from Dip-subtilopeptidase A eliminated methionine as a possibility, and, since performic acid oxidation did not cause any charge difference in the pattern of phosphoryl peptides subjected to electrophoresis at pH3.5, residue X could not be cysteine or cystine. Therefore the possible amino acids were tyrosine, phenylalanine and proline.

In an attempt to distinguish between these possibilities for the identity of residue X, peptide 3 was chromatographed together with a number of amino acids and dipeptides so as to apply the equation of Pardee (1951), as described by Larner & Sanger (1965). The results of these investigations, by using solvents A, B and C (see the Materials and Methods section), suggested that residue X was tyrosine.

This conclusion was then strongly supported by electrophoretic examination of Ser(P)-X (band 3) at pH12, in Na_3PO_4 solution. The tracing of this electrophoresis run is included in Fig. 2, and it can be seen that peptide 3 is no longer slower-moving than Ser(P)-Val and Ser(P)-Ile, as it was at both pH4.7 and

at 8.9; instead it is in line with the lighter dipeptide Ser(P)-Ala. By comparison, the dipeptide Ser(P)-Glu moves slightly faster than Ser(P)-Gly, the extra negative charge overcoming the weight difference. If the relative mobilities are considered on a molecular-weight-per-net-charge basis, then ionization of a tyrosine hydroxyl group would give Ser(P)-Tyr at pH12 a weight-per-net-charge of 87 compared with 85 for Ser(P)-Ala, 95 for Ser(P)-Val and 99 for Ser(P)-Ile. At pH4.7 the weight-per-net-charge for Ser(P)-Tyr is 348, for Ser(P)-Ala 256 and for Ser(P)-Ile 298. The relationship between electrophoretic mobility and charge/mass ratios has been investigated by Offord (1966) for peptides at pH6.5 and 1.9. The relationship at pH3.5, where the pK_a of carboxyl groups will be affected, has not been documented and since the influence of the serine phosphate group (in dipeptides investigated here) will influence the ionization of other charged groups, accurate predictions about the mobility of these dipeptides are not easily made.

Comparison of peptide 3 *with Ser(P)-Tyr.* Final confirmation of the identity of residue X was provided by direct comparison of peptide 3 with a synthetic sample of Ser(P)-Tyr. The synthetic sample was mixed with some radioactive peptide 3 and subjected to electrophoresis at pH3.5, 4.7 and 8.9. Chromatography in solvents A and B was also done with the mixture of synthetic dipeptide and band 3 and in all cases the radioactivity was co-extensive and co-maximal with the ninhydrin stain for the synthetic product.

Additional components of the ^{32}P-labelled partial acid hydrolysate

Residue Y has not been identified beyond the fact that it must be a neutral amino acid, no charge differences between peptides 3 and 5 being observed. The minor peptide band 2 has not been fully characterized.

Extra radioactive bands were sometimes obtained from partial acid hydrolysates of the [^{32}P]phosphoryl enzymes or peptides, but this could be prevented by addition of phenol to the acid before hydrolysis. The phenol, in large excess over the radioactive peptides, presumably reacts with any oxidant or halogenating agent in the acid.

Active-centre sequences of plant and yeast enzymes

In the initial stages of this work (Shaw & Wells, 1967), partially purified preparations of the plant enzyme phaseolain were used (Wells, 1965) and it was apparent that reactive serine residues within two different sequences were labelled with [^{32}P]Dip-F. Nevertheless, it was possible to study the two sets of phosphoryl peptides on a rational basis, as one of them corresponded to a pattern previously studied in Dip-subtilopeptidase A; that is, the pattern was derived from the sequence Thr-Ser-Met-Ala (Sanger & Shaw, 1960). The other pattern of phosphoryl peptides from partially purified phaseolain corresponded to a new serine-containing sequence. Study of this sequence was facilitated by the finding that a yeast peptidase (purified as described in the Materials and Methods section) gave rise to only the new pattern of phosphoryl peptides.

Subsequent further purification of phaseolain resulted in the removal of an endopeptidase, proteinase *b* (Wells, 1968), which was shown to contain an active serine residue within the sequence Thr-Ser-Met-Ala. The homology with subtilopeptidase A does not extend beyond the alanine residue, since exposure of the Dip-F-treated bacterial enzyme to 6M-HCl at 37°C for 12h gives rise to the di-isopropyl form of the above tetrapeptide (Sanger & Shaw, 1960), whereas similar treatment of the plant enzyme yielded only larger phosphoryl peptides (D. C. Shaw and J. R. E. Wells, unpublished work).

Plate 2 shows that the patterns of phosphoryl peptides obtained from the yeast peptidase and phaseolain free of proteinase *b* are identical, and therefore the reactive serine residue in both enzymes occurs in the sequence Glu-Ser-Tyr.

We have obtained phaseolain preparations completely free of other proteolytic activities (Carey & Wells, 1970). Highly sensitive assays on a number of polypeptide substrates (Carey, 1971) have shown that it is a true carboxypeptidase containing no traces of aminopeptidase or endopeptidase activity. The same pattern of phosphoryl peptides (Plate 2; first two slots) is again apparent after partial acid hydrolysis of this [^{32}P]Dip-F-labelled enzyme.

The finding (Shaw & Wells, 1967) that phaseolain from a plant source (French beans) and the peptidase from yeast have the same amino acid sequence around the reactive serine residue and that this sequence is different from any other 'serine' enzyme reported is of some evolutionary interest. Our results also show that 'serine' proteases are not necessarily endopeptidases (cf. subtilopeptidase A, trypsin, chymotrypsin, thrombin, elastase) and that phaseolain, in being inhibited by Dip-F, is different from the pancreatic carboxypeptidases. (Other differences are also apparent; Carey, 1971.) It seems likely from results on other 'serine' proteinases (Sigler *et al.*, 1968; Blow, 1969; Blow *et al.*, 1969; Wright *et al.*, 1969) that, despite differences in active-centre and total-sequence homology between animal and bacterial proteinases, the same catalytic mechanism exists. It would not be surprising, therefore, to find unique histidine and aspartic acid residues contributing to the strong nucleophilic nature of the active serine residue in the yeast peptidase and the plant carboxypeptidase. It is also possible that the tyrosine

residue in the active-centre sequence described here could be equivalent to tyrosine-248 of pancreatic carboxypeptidase A, which moves (on binding substrate) to a position such that its phenolic group can donate a proton to the nitrogen atom of the bond being broken (Reeke *et al.*, 1967).

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