Amino Acid Sequence of the Phosphorylation Site of Yeast
(Saccharomyces cerevisiae) Fructose-1,6-bisphosphatase

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Fructose-1,6-bisphosphatase from the yeast Saccharomyces cerevisiae has properties similar to other gluconeogenic fructose-1,6-bisphosphatases, but an unusual characteristic of the yeast enzyme is that it can be phosphorylated in vitro by CAMP-dependent protein kinase. Phosphorylation also occurs in vivo, presumably as part of a signalling mechanism for the enzyme's degradation. To probe the structural basis for the phosphorylation of yeast fructose-1,6-bisphosphatase, we have developed an improved procedure for the purification of the enzyme and then performed sequence studies with the in vitro-phosphorylated protein as well as with tryptic and chymotryptic peptides containing the phosphorylation site. As a result of these studies, we have determined that yeast fructose-1,6-bisphosphatase has the following 24-residue NH2-terminal amino acid sequence: Pro-Thr-Leu-Val-Asn-Gly-Pro-Arg-Arg-Asp-Ser-Thr-Glu-Phe-Asp-Thr-Asp-Ile-Ile-Thr-Leu-Pro-Arg. The site of phosphorylation is located at Ser-11 in the above sequence. The amino acid sequence around the site of phosphorylation contains the sequence -Arg-Arg-X-Ser- associated with many of the better substrates of CAMP-dependent protein kinase. The sequence of residues 6–15 of pig kidney fructose-1,6-bisphosphatase, showing 7 out of 10 residues in identical positions. The yeast enzyme, however, has a dissimilar NH2-terminal region which extends beyond the NH2 terminus of mammalian fructose-1,6-bisphosphatases and contains a unique phosphorylation site.

This process has been termed catabolite inactivation (3). An initial rapid, reversible inactivation of Fru-Pase has been observed (4–6) that appears to be related to phosphorylation of the enzyme. It has been postulated that phosphorylation of Fru-Pase is a necessary signal for the initiation of proteolysis of the enzyme (7–9). A number of lines of evidence support the idea that reversible inactivation and subsequent proteolysis are CAMP-linked events and that fructose 2,6-bisphosphate also is involved. A transient rise in cAMP and fructose 2,6-bisphosphate was observed upon addition of glucose to a gluconeogenic culture (10). In addition, CAMP-deficient mutants did not show catabolite inactivation, even those suppressed by a second mutation having constitutive CAMP-dependent protein kinase activity (11, 12). Furthermore, yeast Fru-Pase was phosphorylated in vivo by CAMP-dependent protein kinase (8, 9, 13, 14), but not by two different CAMP-independent protein kinases (9). Phosphorylation was greatly stimulated by the presence of fructose 2,6-bisphosphate and was also affected by AMP (8, 9, 13).

Interestingly, the increase in yeast cAMP level (and presumably of CAMP-dependent protein kinase) upon addition of glucose has been difficult to interpret, since a hormone-dependent rise in liver cAMP levels is associated with the gluconeogenic state (15). However, the net metabolic result of increased available glucose appears to be the same in both types of cells: a rise in fructose 2,6-bisphosphate, inhibition of Fru-Pase activity, stimulation of fructose-6-P 1-kinase activity, with resultant stimulation of glycolysis and inhibition of gluconeogenesis (16). In view of the apparent physiological importance of the phosphorylation of yeast Fru-Pase, we undertook the present study to sequence the phosphorylation site of the enzyme and to establish its relationship to the rest of the protein molecule.

EXPERIMENTAL PROCEDURES

RESULTS AND DISCUSSION

Enzyme Purification—Attempts to purify yeast (Saccharomyces cerevisiae) Fru-Pase by a previously published method (25) either failed or produced extremely low yields. The purification procedure that we consequently developed has the
Phosphorylation Site of Yeast Fructose-1,6-bisphosphatase

Advantage of using commercially available bakers' yeast cakes as starting material. This readily available material contains substantial amounts of Fru-P_2ase, although about 5-fold less than that of yeast grown in our laboratory on gluconeogenic carbon sources. The method utilizes a 68 °C heating step which results in the removal of many interfering proteins, including phosphatases and proteases. The presence of fructose 1,6-bisphosphate and Mg^{2+} during heating is necessary to stabilize Fru-P_2ase, but heating should be carried out as soon as the substrate is added because of the rapid destruction of fructose 1,6-bisphosphate by enzymes present in the yeast extract. The purification method retains the important final step of specific elution of Fru-P_2ase from phosphocellulose by fructose 1,6-bisphosphate, a technique which has been used in purifying the enzyme from several sources (13, 25-30). The enzyme was purified in the presence of the proteolytic inhibitor PMSF. Attempts to omit PMSF from the buffers during any step of the purification resulted in rapid loss of most of the enzyme activity, but the purified enzyme was stable after removal of PMSF.

The data of Table I summarize the purification of bakers' yeast (S. cerevisiae) to homogeneity. The developed method has many features in common with a recently published procedure for the purification of Fru-P_2ase from the yeast Kluyveromyces fragilis (13). While this work was in progress, Noda et al. (31) reported another method for the purification of yeast Fru-P_2ase from commercial bakers' yeast or from a glucose-treated S. cerevisiae strain.

Yeast Fru-P_2ase Molecular Weight—As we discussed in a previous publication (14), the pure enzyme was observed to migrate as a single band corresponding to a Mr = 40,000 in SDS-polyacrylamide gel electrophoresis and was indistinguishable in size from the form present in trichloracetic acid-treated yeast cells as detected by immunoblotting. Results of size-exclusion studies on the native enzyme were consistent with a tetramer of 40-kDa subunits. This result contrasted with earlier data indicating that the yeast enzyme is a dimer of 57-kDa subunits (25). In the recent report by Noda et al. (31), purified yeast Fru-P_2ase produced a band on SDS-polyacrylamide gel electrophoresis corresponding to 40 kDa, but the authors suggested that this was the product of limited proteolysis from an in vivo form of 57 kDa.

During our yeast Fru-P_2ase purifications, a protein banding in SDS-polyacrylamide gel electrophoresis at a position corresponding to 56 kDa was consistently observed, but fractions containing the 56-kDa band usually eluted after the main peak of Fru-P_2ase activity from the phosphocellulose column. We have studied this 56-kDa material carefully since it seemed likely to be the same material postulated to be Fru-P_2ase as discussed above. We have concluded that it is not Fru-P_2ase based on the following criteria: (a) Fru-P_2ase activity is associated only with the 40-kDa subunit and fractions containing a large proportion of the 56-kDa band always have lower specific activity; (b) antibody specific for the 40-kDa protein does not precipitate 56-kDa material or give positive reaction in immunoblots; (c) mixtures of the two proteins can be largely separated by gel filtration on Sephacryl S-300 with Fru-P_2ase eluting first; (d) cleavage at aspartyl-prolyl peptide bonds (32) of the 40- and the 56-kDa bands indicates that the two polypeptides are not derivatives of the same protein. The 40-kDa band is cleaved into two large pieces indicating the presence of one Asp-Pro peptide bond, while the 56-kDa protein appears to have no aspartyl-prolyl peptide bonds.

Identification of the Phosphorylation Site of Yeast Fru-P_2ase—To identify the site(s) of in vitro phosphorylation, yeast Fru-P_2ase was phosphorylated with the catalytic subunit of cAMP-dependent protein kinase. ^32P-labeled Fru-P_2ase was carboxymethylated and digested with trypsin, and the peptides were separated by reversed phase HPLC (Fig. 1). Essentially all of the radioactivity was associated with a single peak of absorbance at 214 nm. Sequence data were obtained for the purified 16-residue phosphopeptide (Table II, column 1). No identifiable phenylthiohydantoin amino acid derivative was obtained at cycle number 3, suggesting that residue 3 is phosphorylated (33). The phosphopeptide was then subjected to different treatments designed to modify the putative phosphorylated residue. First, the phosphopeptide was treated with alkaline phosphatase and the resulting products were repurified by reversed phase HPLC. About 80% of the Ala_3-absorbing material eluted in a nonradioactive peak about 3 min later than the untreated radioactive peptide, with the released radioactivity eluting at the void time. The alkaline phosphatase-modified peptide gave the same sequence as before except that serine appeared in cycle 3 (Table II, column 2).

As a second approach to identify the phosphorylated residue, we used the fact that, in the presence of strong base, phosphoserine residues undergo elimination of the phosphate group with formation of dehydroalanine, which can be further derivatized to cysteic acid by sulfite addition (21). Base treatment of the isolated tryptic ^32P-peptide followed by reversed phase HPLC resulted in the release of most of the radioactive phosphate to the void time and a 4-min increase in retention time of the resulting nonradioactive peptide (for details see "Experimental Procedures"). Sulfite addition to the latter peptide caused, in turn, approximately a 3-min reduction in the HPLC retention time. As expected, the amino acid sequence of the peptide after sulfite addition remained the same except that cysteic acid appeared in cycle 3 (Table II, column 3). Thus, the peptide contained a single site of phosphorylation located at serine-3. Amino acid composition analyses of the untreated and also of the alkaline phosphatase-digested phosphopeptides showed serine to be present...
Yeast Pro-Thr-Leu-Val-Asn-Gly-Pro-Arg-Arg-Asp-Ser-Thr-Glu-Gly
Kidney Pro-Thr-Leu-Val-Asn-Gly-Pro-Arg-Arg-Asp-Ser-Thr-Glu-Gly

with the sequence of residues 6-15 of pig kidney Fru-Pzase, Thr-Asp-Ile-Ile-Thr-Leu-Pro-Arg) could clearly be aligned with the sequence of residues 6-15 of pig kidney Fru-Pzase, showing 7 out of the 10 residues in identical positions (shaded residues in Fig. 2). The proximity of the P-serine residue to the NH$_2$ terminus of yeast Fru-Pzase was confirmed by direct sequencing (data not shown).

Table IV

<table>
<thead>
<tr>
<th>Protein substrates for cAMP-dependent protein kinase containing the preferred Arg-Arg-X-Ser sequence</th>
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</thead>
<tbody>
<tr>
<td>Substrate (source)</td>
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<tr>
<td>Phosphorylase kinase a-subunit (rabbit skeletal muscle)</td>
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<tr>
<td>Pyruvate kinase (rat liver)</td>
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<tr>
<td>Pyruvate kinase (porcine liver)</td>
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<tr>
<td>Glycogen synthase, site 1a (rabbit skeletal muscle)</td>
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<tr>
<td>Regulatory subunit of type II cAMP-dependent protein kinase (bovine heart)</td>
</tr>
<tr>
<td>6-Phosphofructose-2-kinase (rat liver)</td>
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<tr>
<td>Pyrotamine, site 2 (rabbit skeletal muscle)</td>
</tr>
<tr>
<td>Pyrotamine, site 3 (rabbit skeletal muscle)</td>
</tr>
<tr>
<td>Fructose-1,6-bisphosphatase (S. cerevisiae)</td>
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</tbody>
</table>

and in agreement with the results obtained by automated sequencing (data not shown).

The sequence of residues 7-16 of the P-peptide (Phe-Asp-Thr-Asp-Ile-Ile-Thr-Leu-Pro-Arg) could clearly be aligned with the sequence of residues 6-15 of pig kidney Fru-Pzase, showing 7 out of the 10 residues in identical positions (shaded residues in Fig. 2). The proximity of the P-serine residue to the NH$_2$ terminus of yeast Fru-Pzase was confirmed by direct sequencing of the purified enzyme. Indeed, as shown in Fig. 2 and Table III, automated Edman degradation of yeast Fru-Pzase for 15 cycles yielded a sequence that overlapped with that of the sequenced P-peptide (Fig. 2 and Table III). The same NH$_2$-terminal sequence was also obtained from a large P-peptide (about 8 kDa) isolated after limited digestion of in vitro phosphorylated Fru-Pzase with chymotrypsin (Table III). The phosphorylation site of yeast Fru-Pzase was therefore located at residue 11 from the NH$_2$ terminus within a sequence of 14 amino acid residues which showed no homology with the corresponding region of mammalian Fru-Pzase, followed by a region of strong homology (Fig. 2). In fact, sequence data from several randomly selected HPLC-purified tryptic peptides suggests that the structures of yeast and mammalian Fru-Pzase are highly homologous (35). Thus, yeast Fru-Pzase appears to have a size and structure closely similar to other gluconegotic Fru-Pzases, but the yeast enzyme contains a dissimilar NH$_2$-terminal region which extends beyond the NH$_2$-terminus of mammalian Fru-Pzases. The phosphorylation site of yeast Fru-Pzase is located within the dissimilar region at a position that corresponds to position 2 of mammalian Fru-Pzases (Fig. 2).

Toyoda and Sy (13) have conducted studies of in vitro phosphorylation using purified enzyme from the closely related yeast K. fragilis. Binding stoichiometry and tryptic peptide mapping by thin layer chromatography suggested that the Kluyveromyces Fru-Pzase may have two phosphorylation sites, in contrast to one site found in Saccharomyces Fru-Pzase. Preliminary studies with Fru-Pzase purified from the non-catabolite-inactivated yeast Candida utilis have failed to show phosphorylation either in vitro or in vivo, although the subunit molecular weight of C. utilis Fru-Pzase was observed to be about the same as that of the Saccharomyces enzyme.³ It would be of interest to establish and compare the amino acid sequences of the NH$_2$-terminal regions of these different yeast Fru-Pzases.

In S. cerevisiae Fru-Pzase, the lack of homology at the NH$_2$ terminus and the presence of additional residues compared with mammalian Fru-Pzases supports a model in which the phosphorylation sites in muscle glycogen phosphorilases (36).

The cAMP-dependent phosphorylation site in yeast Fru-Pzase appears to be completely unrelated to the two cAMP-dependent phosphorylation sites which occur in rat liver Fru-Pzase (37, 38). The latter are found near the COOH terminus in a 24-26-residue extension that is not present in other mammalian Fru-Pzases, even in Fru-Pzases from mice and other members of the Muridae family (39). The amino acid sequences around the phosphorylation sites of rat liver Fru-Pzase are atypical of cAMP-dependent sites, the enzyme is a poor substrate for cAMP-dependent protein kinase (33), and phosphorylation appears to have no effect on the kinetic

³ J. Rittenhouse and F. Marcus, unpublished results.
parameters of the enzyme (37, 40).

Unlike the rat liver Fru-P₂ase phosphorylation sites, the phosphorylation site of yeast Fru-P₂ase contains the recognition sequence for cAMP-dependent protein kinase, Arg-Arg-X-Ser-, that is associated with the best substrates for cAMP-dependent protein kinase. Table IV lists proteins, in addition to yeast Fru-P₂ase, known to contain this sequence and the sequence around the phosphorylation site for each. All are excellent substrates for cAMP-dependent protein kinase and in most cases there is substantial evidence that phosphorylation plays a regulatory role in vivo.

An unusual finding is the apparently free NH₂ terminus in yeast Fru-P₂ase, since cytosolic proteins usually are found to have their NH₂ termini blocked. The explanation may be the NH₂-terminal proline residue, a secondary amine, may not be a substrate for the appropriate transferase. We cannot exclude, however, the possibility that limited proteolytic removal of a blocked NH₂-terminal peptide had occurred. As discussed above, the removed peptide (if any) would have to be small since SDS-polyacrylamide gel electrophoresis failed to detect any subunit molecular weight differences between the purified Fru-P₂ase and immunoblotted enzyme from acid-inactivated yeast cells.

A relevant question still to be answered is whether Fru-P₂ase is phosphorylated in vivo at the same site found in vitro and whether additional site(s) of phosphorylation exist in vivo. Preliminary experiments involving peptide mapping of Fru-P₂ase isolated from yeast grown gluconeogenically in the presence of [³²P]phosphate (with and without addition of glucose to the culture for 20 min) show the same pattern of labeled peptides obtained from in vitro-labeled Fru-P₂ase. We plan to extend our studies on the in vivo phosphorylation of Fru-P₂ase and are at present using reversed phase HPLC separation of the phosphorylated and the corresponding unphosphorylated tryptic peptides to measure levels of in vivo phosphorylation at the cAMP-dependent site under a variety of physiological conditions.

Acknowledgments—We thank Steve Latshaw for his expert assistance with protein sequencing and Dr. Robert L. Heinrikson for his critical reading of the manuscript.

Note Added in Proof—We have been informed by Dr. D. Rogers, Genetics Institute, Cambridge, Massachusetts, of his recent unpublished work on the sequence of the gene coding for S. cerevisiae fructose-1,6-bisphosphatase. The sequence codes for 348 amino acids and begins with a proline residue confirming results of his recent unpub-

REFERENCES

Materials — The following materials were purchased from the sources indicated: glucose-6-phosphate dehydrogenase (from yeast, grade I) and glucose-6-phosphate isomerase (from Escherichia coli, grade I), glycylglycinic acid, nicotinic acid, sodium malonate, sodium bicarbonate, 1,6-fructose-2,6-bisphosphate (Sigma F-4757 approx. 75% purity), 1,6-fructose-1,6-bisphosphate from Yeast; fructose 1,6-bisphosphate containing 20% (pH 7.4) and 0.3 mM MgSO_4, 100 mM sodium phosphate buffer, 100 mM guanidine-HCl, 1% sodium citrate, 0.25 M sucrose, and the purified enzyme from Thermo Scientific. Yeast (baker's yeast) was purchased locally and stored at -80°C for up to two months.

EXPERIMENTAL PROCEDURES

1. Extraction — Two bands of baker's yeast were suspended in 400 ml of cold water, frozen and thawed twice. To the cell suspension, 6 ml of 100 mM NaNO_3 and 2 ml of 0.5% solution of tween was added. The extract of all of the suspension was then washed 2 times with 100 ml of chilled 0.05 M sodium glass beads homogenized in a Waring blender at medium speed (setting STIR) for 3 min. The supernatants were then filtered through a 0.45 μm filter. The resulting supernatant was then added to the beads and the homogenization and decantation process repeated until all of the yeast was homogenized. Finally the beads were washed with 300 ml of 2% Triton X containing 0.1% (wt/vol) MAP and the wash added to the homogenate. The crude homogenate was centrifuged for 60 min at 11,000 g. The resulting supernatant could be stored at -80°C for at least two months.

2. Fast and protamine salt treatment — The supernatant was adjusted to pH 7.5 with Tris base (100 mM). The precipitate that formed was removed by centrifugation. The supernatant was then adjusted to 15.8 M by fructose-1,6-bisphosphate and 1.8 M by the addition of 1.0 M MgSO_4. The precipitate that formed was removed by centrifugation. The supernatant was then adjusted to pH 7.0 with glacial acetic acid and heated at 37°C in a boiling water bath. A temperature of 5°C was reached within 40 seconds and the homogenate was added to an ice-cold solution of 10% (wt/vol) in a final volume of approximately 50 ml. The resuspended pellet was dialyzed for 24 h at 4°C in a half liter dialysis bag (4000 MWCO) wash added to the pooled samples. The crude homogenate was centrifuged for 90 min at 11,000 g. The supernatant could be stored at -80°C for at least two months.

3. Purification of fructose-1,6-bisphosphate — The supernatant was adjusted to pH 7.5 with Tris base (100 mM). The precipitate that formed was removed by centrifugation. The supernatant was then adjusted to 15.8 M by fructose-1,6-bisphosphate and 1.8 M by the addition of 1.0 M MgSO_4. The precipitate that formed was removed by centrifugation. The supernatant was then adjusted to pH 7.0 with glacial acetic acid and heated at 37°C in a boiling water bath. A temperature of 5°C was reached within 40 seconds and the homogenate was added to an ice-cold solution of 10% (wt/vol) in a final volume of approximately 50 ml. The resuspended pellet was dialyzed for 24 h at 4°C in a half liter dialysis bag (4000 MWCO) wash added to the pooled samples. The crude homogenate was centrifuged for 90 min at 11,000 g. The supernatant could be stored at -80°C for at least two months.

4. Phosphocellulose chromatography — After dialysis the material was clarified by centrifugation, diluted twice with calcium buffer, and applied to a column (2.5 x 80 cm) of phosphocellulose. The column was washed with 60 ml of calcium buffer containing 25% (vol/vol) glycerol. The protein concentration of the effluent was monitored by absorbance at 214 nm. The material was then dialyzed with calcium buffer containing 25% (vol/vol) glycerol, then with 200 mM sodium phosphate buffer containing 25% (vol/vol) glycerol, then with 250 mM sodium phosphate buffer. The purified enzyme was recovered by elution with 250 mM sodium phosphate buffer containing 25% (vol/vol) glycerol, then with 100 mM potassium phosphate buffer containing 25% (vol/vol) glycerol, then with 100 mM sodium phosphate buffer containing 10% (vol/vol) glycerol.

5. Alkaline phosphatase treatment — The supernatant was adjusted to pH 9.0 with calcium hydroxide. The supernatant was then adjusted to 15.8 M by fructose-1,6-bisphosphate and 1.8 M by the addition of 1.0 M MgSO_4. The precipitate that formed was removed by centrifugation. The supernatant was then adjusted to pH 7.0 with glacial acetic acid and heated at 37°C in a boiling water bath. A temperature of 5°C was reached within 40 seconds and the homogenate was added to an ice-cold solution of 10% (wt/vol) in a final volume of approximately 50 ml. The resuspended pellet was dialyzed for 24 h at 4°C in a half liter dialysis bag (4000 MWCO) wash added to the pooled samples. The crude homogenate was centrifuged for 90 min at 11,000 g. The supernatant could be stored at -80°C for at least two months.

6. Ion-exchange chromatography on phosphocellulose — The enzyme was dialyzed against 200 mM sodium phosphate buffer containing 25% (vol/vol) glycerol and then centrifuged for 60 min at 11,000 g. The supernatant was then adjusted to pH 7.5 with Tris base (100 mM). The precipitate that formed was removed by centrifugation. The supernatant was then adjusted to 15.8 M by fructose-1,6-bisphosphate and 1.8 M by the addition of 1.0 M MgSO_4. The precipitate that formed was removed by centrifugation. The supernatant was then adjusted to pH 7.0 with glacial acetic acid and heated at 37°C in a boiling water bath. A temperature of 5°C was reached within 40 seconds and the homogenate was added to an ice-cold solution of 10% (wt/vol) in a final volume of approximately 50 ml. The resuspended pellet was dialyzed for 24 h at 4°C in a half liter dialysis bag (4000 MWCO) wash added to the pooled samples. The crude homogenate was centrifuged for 90 min at 11,000 g. The supernatant could be stored at -80°C for at least two months.

7. Amino acid sequence analysis — Automated Edman degradation was performed on Applied Biosystem Model 477A gas-phase sequenator using the standard sequence program and the reagents provided by the manufacturer. Twenty carboxypeptidase A and carboxypeptidase Y digests liberated after each degradation cycle were identified and quantitated as such by reversed-phase HPLC using o-phenylenediamine derivatives (22). The reaction was performed using a Waters Model 600 Liquid chromatograph equipped with an LDC octadeyl-xylenex (4.6 x 25 cm).

Amino acid analysis. These were performed according to the procedure of Wetmore et al. at pH 2.5. (100-200 mg) were hydrolyzed in vacuum at 110°C for 22 h. After hydrolysis the hydrolysate was dehydrated at pH 2.5. The resulting phenylalanyl amino acids were derivatized with phenylisothiocyanate. The resulting phenylalanyl amino acids were derivatized with phenylisothiocyanate. This radioactive peptide was isolated by reversed-phase HPLC on a Waters Model 600 Liquid chromatograph equipped with a Waters Pico-Tip column (3.9 x 5 mm).

<table>
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<th>Error</th>
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* Sample preparation, sequence analysis, and residue identification was performed as described under Experimental Procedures. Amino acids are given in parentheses. 1. The sequence of the sample was determined by Edman degradation with 4 samples mixed and the sequences of the sample were determined by sequence analysis in experiments A and B. The sequences were compared and the sequences were determined by sequence analysis. The abbreviation % denotes cysteic acid.