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A Tetrameric Iron Superoxide Dismutase from the Eucaryote *Tetrahymena pyriformis**

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An iron-containing superoxide dismutase has been purified from the protozoan *Tetrahymena pyriformis*. It has a molecular weight of 85,000 and is composed of four subunits of equal size. The tetramer contains 2.5 g atoms of ferric iron. Visible absorption and electron spin resonance spectra closely resemble those of other iron-containing superoxide dismutases. The amino acid sequence of the iron superoxide dismutase was determined. Each subunit is made up of 196 residues, corresponding to a molecular weight of 22,711. Comparison of the primary structure with the known sequences of other iron-containing superoxide dismutases reveals a relatively low degree of identity (33–34%). However, a higher percentage identity is found with mammalian manganese-containing superoxide dismutases (41–42%). The amino acid sequence is discussed in consideration of residues that may distinguish iron from manganese or dimeric from tetrameric superoxide dismutases.

Superoxide dismutases (SOD; EC 1.15.1.1)[1] containing iron as the active center have been isolated from a wide range of prokaryotes. In one group the protein has a molecular weight of about 40,000 and is composed of two subunits (identical with respect to size) and contains 1–2 g atoms of iron. This is the case for SODs isolated from *Escherichia coli* (1), *Photobacterium leiognathi,* and *Photobacterium sepia* (2), *Pseudomonas ovalis* (3), *Bacillus megaterium* (4), and the facultative anaerobe *Thiobacillus denitrificans* (5); from anaerobes such as *Bacteroides fragilis* (6), purple sulfur bacteria, *Chromatium vinosum* (7), green sulfur bacteria, *Chlorobium thiosulfatophilum* (8), strict anaerobes, *Desulfovibrio desulfuricans* (9), and from blue green alga, for example *Plectonema boryanum* (10, 11) and *Anacystis nidulans* (12). This type of iron enzyme has also been isolated from plant eukaryotes such as *Brassica campestris* (mustard), *Ginkgo biloba,* and *Nuphar luteum* (13, 14) and the protozoan green alga *Euglena gracilis* (15).

A second group of iron enzymes with a molecular weight of about 80,000–90,000, consisting of four equal subunits and with 2–4 g atoms of iron has also been described. This form of superoxide dismutase has been isolated from three prokaryotes, *Mycobacterium tuberculosis* (16) and the strict anaerobe archaebacteria *Thermoplasma acidophilum* (17) and *Methanobacterium bryantii* (18).

In the present study we present the purification and characterization of this tetrameric form of iron superoxide dismutase from the eukaryote *Tetrahymena pyriformis*. Whereas the evolutionary significance of the nature of the metal (iron, manganese, or copper) at the active center seems relatively clear with respect to a hierarchy (in the order given) of superoxide dismutases, the sense of dimeric or tetrameric forms of the iron (or manganese) enzymes is unknown. In addition, although no copper-zinc SOD has so far been detected or isolated in lower eukaryotes, this form of SOD is present in the prokaryote *Photobacterium leiognathi* (19), *Caulobacter crescentus* (20), and some pseudomonads (21).

EXPERIMENTAL PROCEDURES AND RESULTS[2]

Growth of Protozoa—T. pyriformis was grown in a medium containing per liter: iron citrate, 33.5 mg; magnesium sulfate, 246 mg; calcium chloride, 7.3 mg; potassium dihydrogen phosphate, 68 mg; yeast extract, 7.5 g; protease peptone (Difco), 2.5 g; Bactopeptone (Difco), 5.0 g; and glucose (sterilized separately), 15 g. Ten-liter cultures were seeded with 1 liter of preculture (grown for 24 h) and incubated at 28 °C with slow stirring (120 rpm) with an aeration of 6 liters/min. Growth was continued for 48 h to a maximal density of 2,000 cells/mm^3. The protozoa were harvested by centrifugation at about 1,200–2,000 g (7,000–10,000 rpm in a Sharples centrifuge). The cells were then washed once with 500 ml of 50 mM glycine buffer, pH 8.6, and stored frozen at −20 °C.

Enzyme Purification—The protozoa (800 g wet weight) were lysed by homogenization in a mixer with 800 ml of 25 mM glycine buffer, pH 8.6, containing 0.1 mM EDTA and 0.4 mM phenylmethylsulfonyl fluoride (Sigma) for 2 min. To the lysate was added 500 g of DE-52 (Whatman, equilibrated in 1 M glycine buffer, pH 8.6, washed with water to about 1 mM eluant), and the mixture slowly stirred for 10 min. The resin was separated by centrifugation at 7,000 × *g* for 10 min and then washed with 600 ml of 25 mM glycine buffer, pH 8.6,

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[1] The abbreviation used is: SOD, superoxide dismutase.

[2] Portions of this paper (including "Experimental Procedures," part of "Results," Fig. 5, and Tables III–IX) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

containing 0.1 mM EDTA, stirring for 10 min. The mixture was centrifuged for 10 min at 7,000 × *g* and the supernatants, containing the enzyme, were collected. Washing was continued if necessary until negligible activity was present in the supernatant. Ammonium sulfate was added to the combined supernatants to 45% saturation. The precipitate was removed by centrifugation and discarded. The supernatants were adjusted to 85% saturation with ammonium sulfate. Precipitated protein was collected by centrifugation for 30 min at 16,000 × *g*, dissolved in 10 mM acetate buffer, pH 7.0, and dialyzed against the same buffer.

The solution was passed through a column of 600 g of CM-52 (Whatman) equilibrated in 10 mM acetate, pH 7.0, washing with the same buffer. Active fractions (opalescent) were combined and protein precipitated at 85% saturation of ammonium sulfate. The centrifuged precipitate was dissolved in 25 mM Tris acetate, pH 7.0, and dialyzed against this buffer. After dialysis the solution was centrifuged at 16,000 × *g* for 30 min to remove a gelatinous precipitate and then adjusted to pH 8.5 with dilute ammonium hydroxide. This fraction was purified by chromatofocussing using 260 ml of PBE 94 (Pharmacia LKB Biotechnology Inc.) equilibrated in 25 mM Tris acetate, pH 8.5. A major band of activity was eluted at pH 7.0 using a suitable polybuffer solution as eluant. Active fractions were grouped and protein precipitated by addition of ammonium sulfate to 85% saturation. The precipitate was dialyzed against 10 mM acetate, pH 7.0, and then adjusted at pH 6.0.

Final purification was achieved by chromatography on CM-Sepharose (Pharmacia). The dialyzed enzyme (at pH 6.0) was loaded on a column of 50 ml of CM-Sepharose equilibrated with 10 mM acetate, pH 6.0. Elution with a gradient of 10 mM acetate, pH 6.0 (250 ml), to 0.1 M acetate containing 0.1 mM NaCl, pH 6.0 (250 ml), gave a major peak of activity with two minor peaks. The major peak contained 20 mg of protein with an optical density ratio 280/260 = 1.89 and a specific activity of 1097 nitroblue tetrazolium units/mg.

The progress of purification is summarized in Table I.

Protein Determination—During purification protein concentration was determined by the method of Lowry *et al.* (22), using *E. coli* Fe-SOD, *P. leiognathi* Fe-SOD, *E. coli* Mn-SOD, and bovine Cu,Zn-SOD as standards. On the pure enzyme preparation, protein concentration was determined by fringe interferometry.

Enzyme Assays—Superoxide dismutase activity was assayed by the nitroblue tetrazolium method (23).

Determination of pI—Electrofocalization was used mainly to follow purity of the enzyme preparation, since in this technique multiple bands are observed even when a single band appears on gel electrophoresis. The isoelectric point of the purified enzyme was determined on a column of PBE 94 equilibrated at pH 8.5 with 25 mM Tris acetate and eluted with polybuffer 96, pH 6.0. The peak of enzyme activity was eluted at pH 7.0. Gel electrofocussing of a crude extract showed a band of activity at pH 7.0. The isoelectric point of *T. pyriformis* Fe-SOD is more basic than prokaryote Fe-SODs (24). After storage of the crude extract, repetition of the electrofocalization showed the appearance of four to six new bands of activity with pI values between 6.2 and 6.8. This is probably due to proteolytic degradation or deamidation of the enzyme during storage.

TABLE I

Purification of iron superoxide dismutase from T. pyriformis

Experimental details are given in the text. Enzymic activity of the lysate before ammonium sulfate precipitation could not be measured directly by any of the assay techniques for superoxide dismutase. At steps 1 and 2, protein could not be meaningly determined due to presence of extremely opalescent milky solutions (even after centrifugation) containing gelatinous material. NBT, nitroblue tetrazolium.

Fraction	Total activity	Protein	Specific activity	Recovery
	NBT units	*mg*	*units/mg*	%
1. Ammonium sulfate (45–85%)	86,020			(100)
2. CM-52	38,829			45
3. PBE chromatofocussing (ratio 280/260 = 1.06)	32,038	105.3	304	37
4. Ammonium sulfate (85%) (ratio 280/260) = 1.5	25,092	40.7	615	29
5. CM-Sepharose, main peak (ratio 280/260 = 1.89)	22,153	20.2	1,097	26

Molecular Weight—The molecular mass of the native enzyme was determined by gel filtration on columns of Sephadex G-200 and Sephadex G-100 (Pharmacia) using yeast alcohol dehydrogenase (150 kDa), horse liver alcohol dehydrogenase (73 kDa), *E. coli* Mn-SOD (45 kDa), bovine Mn-SOD (85 kDa), human Mn-SOD (85 kDa), and bovine Cu,Zn-SOD (33.5 kDa) as molecular mass markers. Subunit molecular weight was determined by gel electrophoresis in sodium dodecyl sulfate (25) using cytochrome *c*, yeast alcohol dehydrogenase, bovine Cu,Zn-SOD, and *E. coli* Mn-SOD as markers. Since *T. pyriformis* Fe-SOD eluted with the same elution volume of human and bovine Mn-SODs on separate runs of the Sephadex columns and assuming that, for this class of proteins, molecular sizes under these conditions can be correlated with molecular masses, a value of 85,000 Da can be deduced for native *T. pyriformis* Fe-SOD. Similarly, a value of 21,600 Da was obtained for the subunit. *T. pyriformis* Fe-SOD is therefore a tetramer composed of four identical subunits.

Spectroscopic Characterization—Electron paramagnetic resonance spectra were recorded at 77 K with a Varian E-109 X Band spectrometer with 100-KHz field modulation, interfaced to a Minc 11 Digital computer. The microwave power was 10 mW and the frequency 9.18 GHz. Field modulation was 5–20 G. A solution of the stable radical 1,1-diphenyl-2-picrylhydrazyl in benzene was used as a field marker. Ferric iron concentration in the protein solution was determined by a computerized comparison of the integrated spectral surface for the enzyme and for solutions of ferric EDTA complexes (12.5, 125 μg iron/ml) in 1.3 or 13 mM EDTA at pH 7.0, which give an EPR resonance at $g = 4.29$. An appropriate blank within the EPR cavity was subtracted.

The EPR spectrum of *T. pyriformis* superoxide dismutase shows the presence of iron in a high spin ($S = 5/2$) ferric form. The observed *g* values were 4.82, 4.09, and 3.77 (Fig. 1), which arise from the middle Kramer's doublet ($S = \pm 3/2$). There is thus a deviation from a completely rhombic symmetry at the iron atoms with $E/D = 0.247$ using the formulae developed by Wickman *et al.* (26) to determine the relative contributions of the axial/rhombic distortions. At 77 K the resonances arising from the lowest Kramer's doublet ($S = \pm 1/2$), $g = 9.4$, 1.5, and 1.0, could not be detected. The observed line shapes and line widths, respectively, 50, 65, and 90 G, did

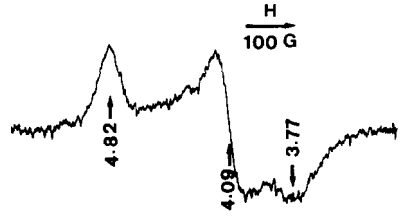

FIG. 1. **Electron spin resonance spectrum at 77 K of *T. pyriformis* superoxide dismutase (6.5 mg/ml in water).**

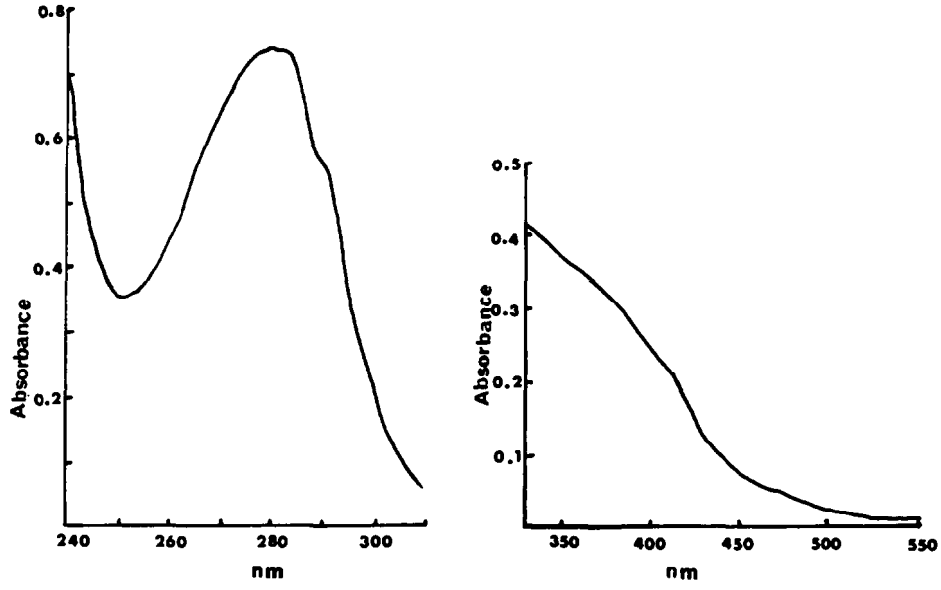

FIG. 2. **Ultraviolet (*left*, 0.325 mg/ml) and visible (*right*, 6.5 mg/ml) absorption spectra of the *T. pyriformis* superoxide dismutase in water.**

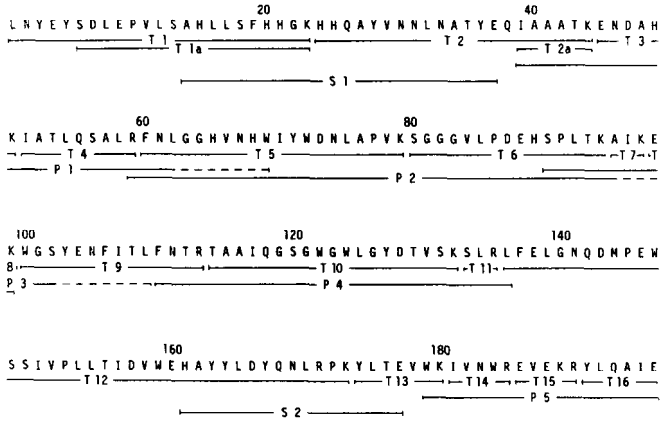

FIG. 3. **Complete amino acid sequence of *T. pyriformis* iron superoxide dismutase.** ——, extent of the various fragments used to construct the sequence; - - -, sequences inferred from amino acid compositions. *T*, tryptic peptides; *P*, peptic peptides; *S*, *S. aureus* V-8 protease peptides.

not indicate structural inequivalence among the iron atoms.

The spectrum is similar to those of other iron containing SODs (1–3, 9, 11, 12, 18, 27) for which *E/D* values range from 0.22 to 0.25.

Determination of ferric iron concentration of a solution of the enzyme at 6.5 mg/ml gave a value of 10.7 μg/ml. Using a molecular weight of 85,000 this indicates 2.5 g atoms of iron/molecule (four subunits) of enzyme.

Metal Identification—Activity of the enzyme was undiminished in 5 mM KCN, indicating the absence of copper at the active site. On the other hand activity was totally destroyed by 2 mM H_2O_2 at room temperature for 20 min, indicating the presence of iron but not of manganese (28). This was confirmed by the visible absorption spectrum (Fig. 2), which was typically that of all other iron SODs with an $\epsilon_{350} = 4865$ and $\epsilon_{400} = 3152$. The ultraviolet absorption spectrum was similar to that of the tetrameric Fe-SODs with a maximum at about 280 nm and a specific absorbance of 2.28/mg/ml (1-cm path length) in comparison with a value of 2.17 reported for *Thermoplasma acidophilum* Fe-SOD (17).

The presence of iron was qualitatively demonstrated by staining polyacrylamide electrophoresis gels of the enzyme with *o*-phenanthroline in the presence of a reducing agent (hydrazine sulfate). A red band appeared at the zone of SOD corresponding to activity or protein stained bands in control gels (29). Estimation of iron was also performed by atomic absorption spectroscopy using a sample dialyzed against distilled water, lyophilized, dried over phosphorous pentoxide, and weighed. The value found was 2.3 g atoms of iron/molecule of 85,000 Da. The enzyme was also examined for the presence of manganese and copper; both were very low and at the limits of detection.

Stability—The enzyme is completely stable between pH 5.5 and 10.5 at 4 °C in 0.1 M buffers for at least 3 days. At pH 5.0 activity drops to about 50%, and at pH 4.5 inactivation is very rapid with total loss within 2 h. With respect to temperature, *T. pyriformis* Fe-SOD is stable at 70 °C for at least 6 h at pH 7 in buffers with an ionic strength of 0.01 or 0.1 M. In 1 mM buffer at 70 °C about 50% loss of activity occurs in 3 h. The same decrease of activity at 3 h is observed in 0.01 M buffer at 75 °C, whereas in 0.1 M buffer at this temperature the loss is about 15%. The enzyme is therefore remarkably stable, particularly at higher ionic strength.

Apoprotein Preparation—Apoprotein was prepared by dialysis of the enzyme against 50 mM acetate, 2 mM EDTA, pH 3.3, followed by dialysis against water. A low recovery of activity was obtained only by dialysis against 0.5 mM ferrous sulfate in 50 mM acetate, pH 6.0. When manganese or copper salts were used instead of ferrous sulfate no activity was obtained.

Sequence Determination—The primary structure of *T. pyriformis* Fe-SOD is shown in Fig. 3. It was determined by sequencing the complete set of peptides generated by cleavage with trypsin; the ordering of these peptides was obtained with overlapping peptides generated from other digests (*Staphylococcus aureus* V-8 protease and pepsin). The peptide nomenclature is also given in Fig. 3. Details on peptide isolation, determination of amino acid compositions and NH_2-terminal sequence for individual peptides are given in the Miniprint. The results obtained after automated Edman degradation of the native protein gave indications of multiple sequences, thus suggesting the occurrence of some proteolytic cleavage of the protein during the purification procedure (see above). This fact also gives an explanation for the isolation of peptide fragments apparently generated by anomalous cleavages, when considering the specificity of the proteolytic enzyme used to digest the protein (see Fig. 3).

DISCUSSION

An iron containing superoxide dismutase has been isolated from *T. pyriformis*. Since the major superoxide dismutase activity in crude extracts from this organism appeared to be exclusively Fe-SOD, it is suggested that *T. pyriformis* contains an iron enzyme rather than a Mn-SOD. However, the presence of traces of the latter enzyme cannot be excluded.

T. pyriformis Fe-SOD has a molecular weight of 85,000 and is composed of four identical subunits. The tetramer contains 2.5 g atoms of ferric iron. Visible absorption and electron spin resonance spectra closely resemble those of other iron containing superoxide dismutases.

The amino acid sequence of the subunit has been determined and is reported in Fig. 3. It is composed of 196 amino acid residues, corresponding to a molecular weight of 22,711. The automated Edman degradation of the apoprotein gave multiple sequences, indicating the presence of several molecular forms differently truncated, mainly at the NH_2 terminus of the protein. This is probably due to proteolytic degradation of the enzyme during the purification procedures and is in accord with the appearance of multiple bands of enzyme activity in electrofocussing experiments. The more extended NH_2-terminal version of the enzyme, whose sequence starts from Leu-1 (Fig. 3), represents only a small percentage (11%) of the total protein molecules present in our preparation, whereas the prevalent molecular species (50%) has Ser-6 as the first residue. Other molecular species have Glu-9, Ile-39, and Asp-47 as NH_2-terminal residue, respectively. It cannot be excluded that the true NH_2-terminal peptide of the native protein has been lost during purification.

The amino acid sequence of Fe-SOD from *T. pyriformis* is compared in Fig. 4 with the sequences of other iron and manganese containing superoxide dismutases. A number of gaps have been inserted into the sequences to optimize the alignment. The degree of sequence identity was calculated on the basis of alignments shown in Fig. 4, with common gaps excluded from the calculations. The results have been compiled in Table II. The overall degree of identity between the 11 sequences reported in Fig. 4 is 15.5%, whereas the percentage identity between the iron enzymes is 26.4%. If the sequence of *T. pyriformis* enzyme is compared with the sequences of Mn-SODs reported in Fig. 4, 19.2% identity is found. However, if the sequence of *T. pyriformis* Fe-SOD is compared with the mammalian Mn-SODs, the percentage identity increases to 33.6%, thus suggesting that in the protozoa the Fe-SOD is on the way to becoming a "true" eukaryote Mn-SOD. This means that the global percentage structural identity of the various superoxide dismutase protein moieties is related to the phylogenetic proximity of the source organisms more than being influenced by features such as the occurrence of either iron or manganese at the active site and the assembling of either a dimeric or a tetrameric structure.

Crystallographic data available for a number of both Fe- and Mn-SODs have revealed extensive structural similarities of the two classes of isoenzymes (41–44). In particular, identical residues were assigned as ligands either to the iron or to the manganese, and these residues are also conserved in *T. pyriformis* (Fig. 4).

Recently, comparative analyses of these crystallographic data and of the available sequence information have been

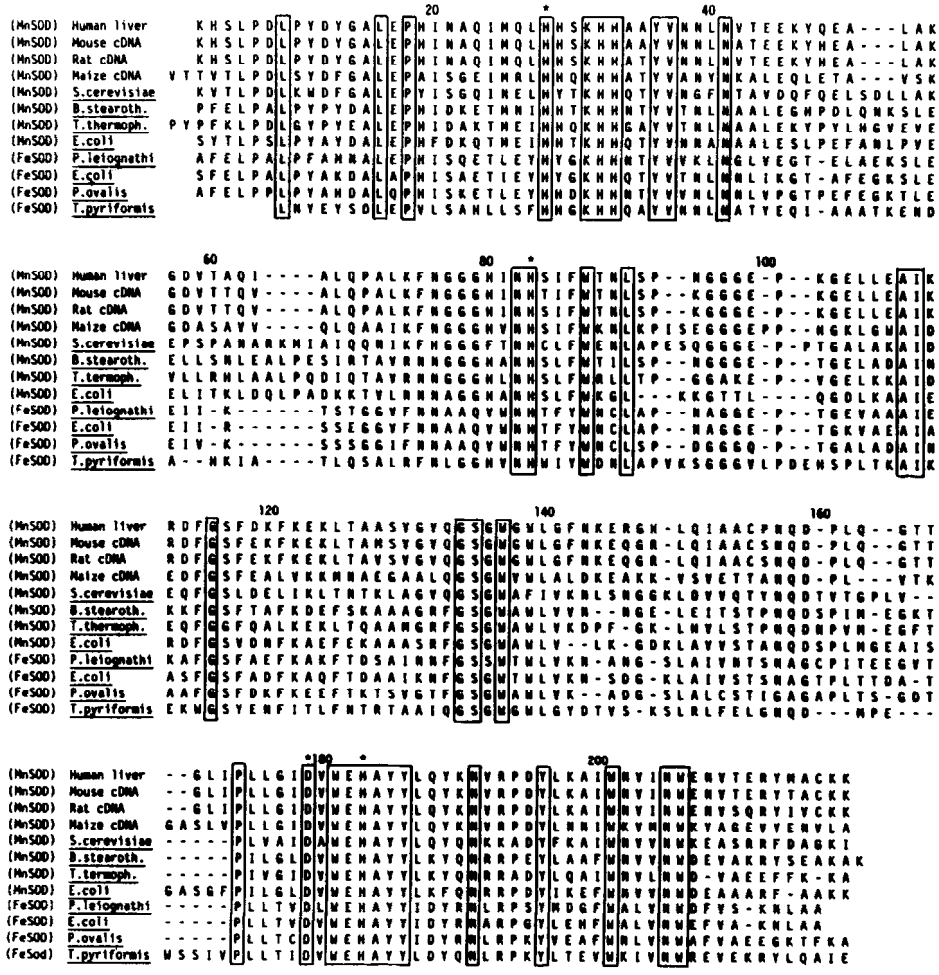

FIG. 4. **Comparison of the amino acid sequences of Mn-SOD from human liver (30), mouse (31), rat (32), maize (33), *S. cerevisiae* (34), *B. stearothermophilus* (35), *T. thermophilus* (36), *E. coli* (37), and Fe-SOD from *P. leiognathi* (38), *E. coli* (39), *P. ovalis* (40), and *T. pyriformis*.** Gaps were introduced to maximize the homology. *Boxes* indicate positions at which residues are identical. Metal ligands are indicated with an *asterisk*.

TABLE II

Sequence identities between Fe- and Mn-superoxide dismutases

Values are given as percentage of sequence identities.

	Manganese							Iron			
	Mouse	Rat	Maize	*S. cerevisiae*	*B. stearothermophilus*	*T. thermophilus*	*E. coli*	*P. leiognathi*	*E. coli*	*P. ovalis*	*T. pyriformis*
Manganese											
Human liver	93.94	92.93	54.85	45.28	50.00	49.76	42.92	37.56	38.54	42.16	41.42
Mouse cDNA		94.45	54.85	43.87	48.08	48.33	42.45	37.56	39.02	42.65	41.98
Rat cDNA			55.34	43.87	48.08	47.37	42.45	37.56	38.05	41.67	40.57
Maize cDNA				44.70	40.93	42.99	38.89	30.00	31.90	32.70	40.65
S. cerevisiae					40.00	41.23	39.72	34.45	33.65	33.97	34.11
B. stearothermophilus						63.33	59.15	49.51	49.51	51.94	38.25
T. thermophilus							51.64	37.02	38.46	41.35	34.86
E. coli								39.15	41.15	42.18	37.04
Iron											
P. leiognathi									75.13	64.47	33.97
E. coli										66.50	33.02
P. ovalis											33.02

performed by various authors with the aim of providing a structural explanation for distinctive properties of the two isoenzymes, such as the metal cofactor specificity or differences in their solution properties. Thus, Isobe *et al.* (45) and Parker and Blake (46) have focussed their attention on residues at the active site potentially responsible for metal selectivity. For instance, by observing the residues occurring within a sphere of 10-Å radius from the metal cofactor, 5 of them were classified by Parker and Blake (46) as primary discriminating candidates, since they were found to be totally invariant within each enzyme class. Now, structural data on the iron superoxide dismutase from *T. pyriformis* indicate that four out of these five positions, corresponding to residues 78, 79, 158, and 159, are occupied by Gly, Gly, Gln, and Asp, respectively, observed previously only in Mn-SODs. Thus, we should consider either that residues occupying these positions are not responsible for conferring the distinguishing properties of the metal binding specificity or that "discriminating residue rules" may hold for prokaryotic Fe-SODs versus pro- and eukaryotic Mn-SODs, but not for eukaryotic Fe-SODs. The acquisition of sequence information from other eukaryotic Fe-SODs will help in clarifying this point.

On the other hand the primary structure of *T. pyriformis* Fe-SOD seems to confirm the "discriminating" function performed at the active site by position 86, which is occupied by Tyr in Fe-SODs and by Phe in Mn-SODs. Recently, on the basis of the proximity of Tyr-76 and Tyr-173 in the *E. coli* Fe-SOD (Tyr-86 and Tyr-196 in Fig. 4), Beyer *et al.* (47) suggested that, at the high pH selectively requested for the apo preparation from the iron enzyme of *E. coli,* ionization of these tyrosine residues would result in an electrostatic repulsion between the two phenolate moieties. An equivalent repulsion would not be observed in the Mn-SOD from *E. coli,* and presumably in the other Mn-SODs, due to the replacement of Tyr-86 with Phe. The corresponding changes in the cavity environment should account for the substantial differences observed in solution properties between the two isoenzymes of *E. coli.*

In any case, the results from sequence analysis of *T. pyriformis* Fe-SOD indicate that the assignment of cDNA derived sequences to either Mn- or Fe-SOD class of protein, only on the basis of sequence homology or of the presence of discriminating residues, should be considered with caution.

Moreover, the case of *T. pyriformis* Fe-SOD strengthens the idea that Gly-78, Gly-79, Gln-158, and Asp-159 should be either conserved or substituted in a concerted manner by Ala, Gln, Ala, and Gly, respectively, for reasons independent of the metal selectivity but of more general relevance for the catalytic competence of the metal environment. In this respect, it has been already observed (44) that either glutamine 69 in *E. coli* Fe-SOD (79 in Fig. 4) or glutamine 141 in Mn-SOD (158 in Fig. 4), although from two different domains, may equally function structurally at the active site as a bridge between Tyr-34 and Trp-122 in the same proteins (Tyr-36 and Trp-137, respectively, in Fig. 4).

Since the amino acid sequence of *T. pyriformis* represents the first primary structure known for a tetrameric Fe-SOD, a comparison between this structure and that of *T. thermophilus* Mn-SOD in the region 40–70 involved in the tetrameric stabilization (41) was performed and it indicates different lengths and a high sequence variability. This confirms the suggestion that in the Mn-, Fe-SOD family the formation of tetramers over dimers is species-dependent (30). Since heat stability of both tetrameric Fe-SOD from *T. pyriformis* and Mn-SOD from *T. thermophilus* is greater than that of dimeric Fe-SOD from *P. leiognathi* or Mn-SOD from *Bacillus stearothermophilus* (29), one could suggest that this property is related to the quaternary structure of this class of proteins.

It is also interesting to note the high extent of dissimilarity in the region 68–89, where 14 over 19 residues conserved in the other Fe-SODs are substituted in the primary structure of *T. pyriformis* Fe-SOD. Among these, there is also the replacement of the Cys-89, which was considered as a candidate for the inactivation reaction of Fe-SOD by hydrogen peroxide on the basis of the sequence information previously available (48).

In conclusion, the structural work on *T. pyriformis* confirms once more that sequence information derived from proteins purified from wild-type organisms judiciously chosen at evolutionary crucial positions provides invaluable information in refining the understanding of the structural basis for enzyme function.

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A TETRAMERIC IRON SUPEROXIDE DISMUTASE FROM THE EUKARYOTE TETRAHYMENA PYRIFORMIS

BY

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EXPERIMENTAL PROCEDURES

Materials . Trypsin (code TRTPCK) was from Worthington Biochemical Co.; *Staphylococcus aureus* V-8 protease from Miles and pepsin from Sigma Chemical Co. CNBr was from Fluka and high performance liquid chromatography (hplc) -grade solvents from Farmitalia Carlo Erba; gas-phase sequencer chemicals from Applied Biosystems. All other chemicals were of reagent grade.

Enzymatic and chemical cleavages . 3 mg of apoprotein were suspended in 0.3 ml of 0.1 M ammonium bicarbonate and incubated at 37°C for 3 h with trypsin. The enzyme to substrate (E/S) ratio was 1/30. A second sample (3 mg) of apoprotein was digested in the same conditions reported above with *S. aureus* V-8 protease, and a third aliquot of 2 mg was dissolved in 0.2 ml of 5% formic acid and digested at room temperature for 5 min with pepsin (E/S ratio = 1/50). After digestion, the peptide mixtures were directly injected onto the column for purification by hplc (see below). Cleavage at the single methionyl residue was obtained by incubating overnight 2 nmol of apoprotein in 0.1 ml 70% formic acid with a small crystal of CNBr.

Hplc purification . Peptide purification was achieved by hplc using a Beckman model 332 instrument, on a macroporous reverse-phase column (Aquapore RP-300, 7 x 250 mm, 7 μm, Brownlee Labs) eluted with gradients of 0 to 70% acetonitrile in 0.2 % trifluoroacetic acid (TFA), at a flow rate of 2.5 ml/min. Elution of the peptides was monitored on a Beckman 165 spectrophotometer at 220 and 280 nm.

Analytical techniques . Quantitative amino acid analyses were carried out on 0.5-1 nmol of peptide hydrolyzed in vapor phase with 6 M HCl, containing 0.1% phenol, at 110°C for 24 h. The amino acid composition of hydrolysed peptides and of the protein was determined using an LKB 2131 Alpha plus Instrument.

The amino acid sequences of the apoprotein and peptides was determined by automated Edman degradation using an Applied Biosystems mod. 470A Gas-phase Sequencer equipped with an Applied Biosystems mod. 120A PTH-analyzer for the on-line identification of amino acid derivatives. 0.3-2 nmol of samples were loaded onto a TFA-treated glass fiber filter, coated with polybrene and prewashed according to the manufacturer's instructions.

Peptide nomenclature . The peptides were numbered retrospectively according to their location in the sequence, starting from the N-terminus. Tryptic peptides are designated with a T, *S. aureus* protease peptides with a S, and peptic peptides with a P.

RESULTS

Amino acid composition of the protein . In Table III the amino acid composition of the protein determined after acid hydrolysis is compared with the composition deduced from the sequence.

N-terminal sequence of the protein . The results obtained following automated Edman degradation of the apoprotein are reported in Table IV and clearly show an heterogeneity of the protein sample. The interpretation of these results, on the basis of the possible occurrence of differently truncated molecular forms of the enzyme (see Experimental procedures) was possible after the analysis of the various peptide fragments and reconstruction of the complete sequence.

Tryptic peptides . The elution profile from hplc of the tryptic peptides is reported in Fig. 5. The first peak, eluted immediately after the injection, was further purified by preparative thin layer chromatography on cellulose plates (Merck) developed with BAWP (butanol/acetic acid/water/pyridine, 15/3/12/10, v:v). Other impure fractions were further purified by hplc in slightly different gradient conditions. Analytical data of the complete set of tryptic peptides are summarized in Tables V and VI.

Automated sequencing after CNBr cleavage . The analysis of peptide T12 indicated that the single methionine of the protein was followed by a proline. Therefore, a small sample (2 nmol) of the apoprotein, treated with CNBr and then lyophilized, was directly loaded onto the glass fiber filter of the sequencer. Subsequently, the filter was treated with o-phthaldialdehyde to prevent sequencing of all the peptide fragments except those with an N-terminal proline (49). Automated Edman degradation of this sample gave information from residue 145 to residue 182 (Table VII).

S. aureus protease and peptic peptides. Analytical data on the peptides obtained after digestion with the Glu-specific S. aureus protease and pepsin, which gave information useful for completion of the sequence, are reported in Table VIII and IX, respectively.

TABLE III

Amino Acid Composition of Tetrahymena pyriformis Superoxide Dismutase

Amino acid	Apoprotein	Sequence
Aspartic acid§	20.1	21
Threonine	12.0	10
Serine	11.5	12
Glutamic acid§	21.9	21
Proline	6.6	7
Glycine	13.0	12
Alanine	14.9	15
Half-cystine*	0	0
Valine	10.7	11
Methionine	1.1	1
Isoleucine	9.1	9
Leucine	23.1	23
Tyrosine	11.4	13
Phenylalanine	5.2	5
Histidine	9.2	10
Lysine	11.5	11
Arginine	6.4	6
Tryptophan	ND	9
Total residues		196

Acid hydrolysis was performed on apo-superoxide dismutase for 24 h.
§8 aspartic acid and 13 asparagine residues.
§14 glutamic acid and 7 glutamine residues.
*After performic acid oxidation.
ND, not determined.

TABLE IV

Automated Edman Degradation of T. pyriformis Apoprotein

Cycle no.	PTH-aa (yield, pmol)				
1	Leu[1] (140)	Ser[6] (640)	Glu[9] (126)	Ile[39] (85)	Asp[47] (71)
2	Asn (58)	Asp (127)	Pro (54)	Ala (250)*	Ala (250)*
3	Tyr (70)	Leu (325)	Val (31)	Ala (125)	His (25)
4	Glu (308)*	Glu (308)*	Leu (93)	Ala (132)	Lys (94)
5	Tyr (61)	Pro (118)	Ser (30)	Thr (69)	Ile (85)
6	Ser (73)	Val (181)	Ala (158)*	Lys (83)	Ala (158)*
7	Asp (35)	Leu (183)	His (20)	Glu (72)	Thr (76)
8	Leu (253)*	Ser (115)	Leu (253)*	Asn (33)	Leu (253)*
9	Glu (82)	Ala (145)	Leu (112)	Asp (30)	Gln (38)
10	Pro (39)	His (42)	Ser (62)*	Ala (37)	Ser (62)*
11	Val (63)	Leu (144)	Phe (22)	His (21)	Ala (43)
12	Leu (261)*	Leu (261)*	His (12)	Lys (32)	Leu (261)*
13	Ser (104)*	Ser (104)*	His (13)	Ile (43)	Arg (19)
14	Ala (122)	Phe (89)	Gly (36)	Ala (122)*	Phe (89)*
15	His (25)*	His (25)*	Lys (26)	Thr (50)	Asn (38)
16	Leu (95)*	His (29)*	His (29)*	Leu (95)*	Leu (95)*
17	Leu (32)	Gly (91)*	His (11)	Gln (31)	Gly (91)*
18	Ser (58)*	Lys (56)	Gln (25)	Ser (58)*	Gly (28)
19	Phe (15)	His (20)*	Ala (22)*	Ala (22)*	His (20)*
Percent of the total	11%	50%	8%	12%	14%

* In these cases, the yield accounts for the release of the same PTH-aa from different molecular species present in the mixture.

TABLE V

Amino Acid Composition of Tryptic Peptides

The composition from sequence analysis of each peptide is indicated by the numbers in parentheses

Peptide	T 1	T 1a	T 2	T 2a	T 3	T 4	T 5	T 6	T 7
Residue nos.	1-23	6-23	24-44	39-44	45-50	51-59	60-79	80-94	95-97
Asp	1.9(2)	1.0(1)	2.7(3)		1.8(2)		3.9(4)	0.9(1)	
Thr			1.6(2)	0.9(1)		0.8(1)		0.8(1)	
Ser	2.8(3)	2.9(3)				0.9(1)		1.7(2)	
Glu	1.6(2)	1.0(1)	2.8(3)		1.1(1)	1.0(1)		1.0(1)	
Pro	1.0(1)	0.9(1)					1.0(1)	1.9(2)	
Gly	1.1(1)	1.0(1)					2.1(2)	2.8(3)	
Ala	1.0(1)	0.9(1)	4.4(5)	3.0(3)	1.0(1)	2.1(2)	1.0(1)		1.0(1)
Val	0.9(1)	0.9(1)	1.0(1)				2.0(2)	0.7(1)	
Met									
Ile			1.0(1)	0.6(1)		0.8(1)	0.9(1)		0.7(1)
Leu	4.8(5)	3.8(4)	1.0(1)			1.7(2)	1.9(2)	1.6(2)	
Tyr	1.7(2)		1.6(2)				0.9(1)		
Phe	1.0(1)	1.0(1)					1.0(1)		
His	2.8(3)	3.0(3)	1.6(2)		0.8(1)		1.9(2)	0.8(1)	
Lys	1.0(1)	1.0(1)	1.0(1)	0.9(1)	1.0(1)		1.1(1)	1.0(1)	0.9(1)
Arg						0.8(1)			
Trp							+ (2)		
Yield %	26.1	19.0	38.1	12.3	15.4	77.7	26.2	44.8	13.8

TABLE V (continued)

Peptide	T 8	T 9	T 10	T 11	T 12	T 13	T 14	T 15	T 16
Residue nos.	98-99	100-113	114-132	133-135	136-173	174-180	181-185	186-190	191-196
Asp		2.0(2)	1.0(1)		4.6(5)		1.0(1)		
Thr		1.8(2)	1.7(2)		1.0(1)	0.8(1)			
Ser		0.9(1)	2.1(2)	1.0(1)	2.1(2)				
Glu	1.0(1)	1.0(1)	1.0(1)		4.8(5)	1.0(1)		1.8(2)	2.0(2)
Pro					2.6(3)				
Gly		1.1(1)	3.9(4)		1.1(1)				
Ala			1.8(2)		1.0(1)				1.0(1)
Val			1.0(1)		1.9(2)	0.9(1)	0.9(1)	0.8(1)	
Met					0.9(1)				
Ile		0.9(1)	0.9(1)		1.7(2)		0.8(1)		0.8(1)
Leu		1.0(1)	0.8(1)	0.7(1)	5.5(6)	0.8(1)			1.0(1)
Tyr		1.0(1)	1.0(1)		2.5(3)	0.8(1)			0.9(1)
Phe		1.8(2)			1.0(1)				
His					1.0(1)				
Lys	0.9(1)		1.1(1)		1.1(1)	1.0(1)		0.9(1)	
Arg		1.1(1)		1.0(1)	1.1(1)		1.0(1)	1.0(1)	
Trp		+ (1)	+ (2)		+ (2)	+ (1)	+ (1)		
Yield %	23.1	47.6	42.9	20.0	59.5	57.1	60.6	18.5	42.9

Presence of tryptophan was indicated by absorbance at 280 nm.

TABLE VI

Automated Edman Degradation of Tryptic Peptides

Cycle no.	T 1 (0.5 nmol)		T 1a (0.4 nmol)		T 2 (0.8 nmol)		T 2a (0.3 nmol)		T 3 (1.2 nmol)		T 4 (0.8 nmol)		T 5 (0.5 nmol)		T 6 (1.3 nmol)		T 7 (0.3 nmol)	
	PTH-aa	Yield (pmol)	PTH-aa	Yield (pmol)	PTH-aa	Yield (pmol)	PTH-aa	Yield (pmol)	PTH-aa	Yield (pmol)	PTH-aa	Yield (pmol)	PTH-aa	Yield (pmol)	PTH-aa	Yield (pmol)	PTH-aa	Yield (pmol)
1	Leu	95	Ser	138	His	104	Ile	51	Glu	116	Ile	280	Phe	284	Ser	619	Ala	56
2	Asn	61	Asp	65	His	95	Ala	73	Asn	135	Ala	282	Asn	209	Gly	461	Ile	34
3	Tyr	66	Leu	99	Gln	197	Ala	66	Asp	76	Thr	290	Leu	261	Gly	494	Lys	8
4	Glu	78	Glu	92	Ala	257	Ala	68	Ala	100	Leu	215	Gly	230	Gly	445		
5	Tyr	68	Pro	72	Tyr	198	Thr	30	His	30	Gln	201	Gly	236	Val	449		
6	Ser	63	Val	56	Val	171	Lys	5	Lys	94	Ser	161	His	129	Leu	406		
7	Asp	38	Leu	82	Asn	140					Ala	197	Val	195	Pro	289		
8	Leu	49	Ser	58	Asn	152					Leu	130	Asn	153	Asp	173		
9	Glu	36	Ala	47	Leu	181					Arg	23	His	117	Glu	251		
10	Pro	30	His	16	Asn	178							Trp	129	His	64		
11	Val	22	Leu	28	Ala	200							Ile	166	Ser	186		
12	Leu	38	Leu	30	Thr	157							Tyr	139	Pro	110		
13	Ser	22	Ser	21	Tyr	110							Trp	106	Leu	94		
14	Ala	21	Phe	16	Glu	115							Asp	68	Thr	58		
15	His	13	His	7	Gln	106							Asn	67	Lys	33		
16	Leu	27	His	9	Ile	115							Leu	57				
17	Leu	32	Gly	13	Ala	132							Ala	44				
18	Ser	15	Lys	2	Ala	141							Pro	21				
19	Phe	10			Ala	138							Val	9				
20	His	2			Thr	79							Lys	5				
21	His	9			Lys	47												
22	Gly	9																
23	Lys	3																
Repetitive yield %	93.8 (Leu 8-Leu 12)		89.0 (Leu 3-Leu 7)		96.5 (Ala 4-Ala 11)		96.5 (Ala 2-Ala 4)		86.1 (Asn 2-Ala 4)		93.1 (Ala 2-Ala 7)		94.9 (Asn 2-Asn 8)		82.4 (Pro 7-Pro 12)			
Position	T-23		6-23		24-44		39-44		45-50		51-59		60-79		80-94		95-97	

TABLE VI (continued)

Cycle no.	T 8 (1.5 nmol)		T 9 (1.5 nmol)		T 10 (0.9 nmol)		T 11 (1.3 nmol)		T 12 (1.8 nmol)		T 13 (2.0 nmol)		T 14 (1.2 nmol)		T 15 (1.5 nmol)		T 16 (2.0 nmol)	
	PTH-aa	Yield (pmol)	PTH-aa	Yield (pmol)	PTH-aa	Yield (pmol)	PTH-aa	Yield (pmol)	PTH-aa	Yield (pmol)	PTH-aa	Yield (pmol)	PTH-aa	Yield (pmol)	PTH-aa	Yield (pmol)	PTH-aa	Yield (pmol)
1	Glu	316	Arg	553	Thr	316	Ser	378	Leu	540	Tyr	833	Ile	260	Glu	432	Tyr	666
2	Lys	231	Gly	473	Ala	351	Leu	247	Phe	327	Leu	714	Val	280	Val	316	Leu	528
3			Ser	497	Ala	345	Arg	62	Glu	461	Thr	501	Asn	170	Glu	298	Gln	425
4			Tyr	432	Ile	220			Leu	547	Glu	617	Trp	154	Lys	323	Ala	616
5			Glu	390	Gln	198			Gly	444	Val	427	Arg	35	Arg	119	Ile	383
6			Asn	277	Gly	171			Asn	320	Trp	295					Glu	163
7			Phe	332	Ser	166			Gln	372	Lys	355						
8			Ile	343	Gly	121			Asp	187								
9			Thr	330	Trp	62			Met	542								
10			Leu	327	Gly	103			Pro	319								
11			Phe	268	Trp	41			Glu	320								
12			Asn	210	Leu	100			Trp	279								
13			Thr	273	Gly	87			Ser	298								
14			Arg	65	Tyr	88			Ser	185								
15					Asp	44			Ile	149								
16					Thr	21			Val	140								
17					Val	43			Pro	95								
18					Ser	54			Leu	107								
19					Lys	18			Leu	121								
									Thr	55								
									Ile	94								
									Asp	40								
									Met	52								
									Trp	48								
									Glu	25								
									His	14								
									Ala	68								
									Tyr	57								
									Tyr	69								
									Leu	63								
									Asp	32								
									Tyr	52								
									Gln	38								
									Asn	34								
									Leu	49								
									Arg	49								
									Pro	47								
									Lys	4								
Repetitive yield %	-		96.8 (Phe 1-Phe 11)		92.7 (Gly 10-Gly 13)		-		94.8 (Glu 3-Glu 11)		87.7 (Leu 2-Val 5)		80.9 (Ile 1-Asn 3)		81.9 (Glu 1-Glu 3)		89.9 (Leu 2-Ile 5)	
Position	98-99		100-113		114-132		133-135		136-173		174-180		181-185		186-190		191-196	

TABLE VII

Automated Edman Degradation of CNBr Cleaved Apoprotein

Cycle No	PTH-aa	Yield (pmol)	Cycle	PTH-aa	Yield (pmol)
1	Pro[a]	443	25	Asn	40
2	Glu	447	26	Leu	78
3	Trp	325	27	Arg	24
4	Ser	318	28	Pro[a]	28
5	Ser	190	29	X[b]	
6	Ile	162	30	Tyr	25
7	Val	130	31	Leu	27
8	Pro	95	32	Thr	21
9	Leu	114	33	Glu	13
10	Leu	122	34	Val	23
11	Thr	78	35	X[b]	
12	Ile	87	36	X[b]	
13	Asp	37	37	Ile	35
14	Val	72	38	Val	25
15	Trp	39	39	Asn	18
16	Glu	56	40	X[b]	
17	His	24	41	Arg	12
18	Ala	102	42	Glu	15
19	Tyr	67	43	Val	24
20	Tyr	78	44	Glu	14
21	Leu	79	45	X[b]	
22	Asp	26	46	Arg	13
23	Tyr	70	47	Tyr	12
24	Gln	45	48	Leu	7

Repetitive yield: 97.0% (Leu 9-Leu 21)
Position: 145-196

[a]Before the 1st and after the 27th cycle the filter was treated with o-phthalaldehyde (see text).
[b]X: not identified.

TABLE VIII

Amino Acid Composition of Peptic and S. aureus Protease Peptides

The composition from sequence analysis of each peptide is indicated by the numbers in parentheses

Peptide	P 1	P 2	P 3	P 4	P 5	S 1	S 2
Residue nos.	39-69	59-99	90-109	110-136	179-196	14-37	161-177
Asp	3.7(4)	4.8(5)	1.0(1)	2.0(2)	1.0(1)	2.8(3)	2.0(2)
Thr	1.5(2)	0.9(1)	1.5(2)	2.4(3)		0.9(1)	0.9(1)
Ser	1.1(1)	2.2(2)	1.8(2)	2.8(3)		1.0(1)	
Glu	2.0(2)	2.0(2)	2.0(2)	1.1(1)	3.7(4)	2.2(2)	1.8(2)
Pro		3.0(3)	0.7(1)				0.9(1)
Gly	2.1(2)	4.4(5)	1.1(1)	3.7(4)		1.1(1)	
Ala	5.4(6)	1.7(2)	0.9(1)	2.0(2)	1.1(1)	3.0(3)	0.9(1)
Val	0.8(1)	2.4(3)		0.9(1)	1.8(2)	1.0(1)	
Ile	1.9(2)	1.7(2)	1.4(2)	1.0(1)	1.5(2)		
Leu	3.0(3)	4.0(4)	1.9(2)	2.9(3)	1.0(1)	2.5(3)	2.5(3)
Tyr		0.7(1)	0.8(1)	1.0(1)	0.9(1)	1.4(2)	3.2(4)
Phe	1.0(1)	1.0(1)	1.0(1)	0.8(1)		1.0(1)	
His	2.4(3)	2.4(3)				3.5(5)	0.8(1)
Lys	2.1(2)	3.5(4)	2.7(3)	1.0(1)	2.0(2)	1.0(1)	0.8(1)
Arg	0.8(1)	0.9(1)		1.7(2)	1.7(2)		0.7(1)
Trp	+ (1)	+ (2)	+ (1)	+ (2)	+ (2)		
Yield %	12.4	2.5	10.0	21.5	8.5	17.1	8.5

Presence of tryptophan was indicated by absorbance at 280 nm.

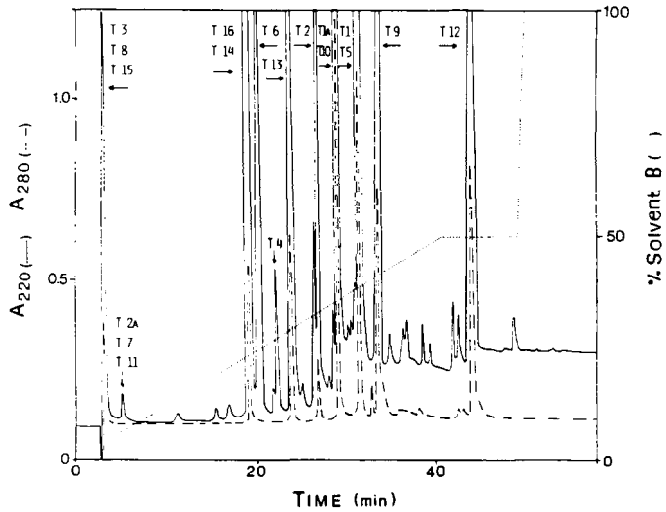

Fig. 5. Reverse phase high performance liquid chromatography of the tryptic digest of apo-protein. Conditions of analysis are reported in the text. The numbers above the peaks refer to the tryptic peptide(s) subsequently found in these peaks.

TABLE IX

Automated Edman Degradation of Peptic and S. aureus Peptides

Cycle No	P 1 (1.2 nmol)		P 2 (0.5 nmol)		P 3 (1.0 nmol)		P 4 (2.0 nmol)		P 5 (1.3 nmol)		S 1 (1.5 nmol)		S 2 (1.0 nmol)	
	PTH-aa	Yield (pmol)	PTH-aa	Yield (pmol)	PTH-aa	Yield (pmol)	PTH-aa	Yield (pmol)	PTH-aa	Yield (pmol)	PTH-aa	Yield (pmol)	PTH-aa	Yield (pmol)
1	Ile	183	Arg	58	Ser	122	Phe	420	Trp	165	Ala	584	His	49
2	Ala	220	Phe	113	Pro	87	Asn	330	Lys	215	His	95	Ala	78
3	Ala	210	Asn	77	Leu	96	Thr	306	Ile	172	Leu	553	Tyr	53
4	Ala	189	Leu	122	Thr	41	Arg	152	Val	139	Leu	470	Tyr	49
5	Thr	106	Gly	91	Lys	53	Thr	290	Asn	104	Ser	308	Leu	53
6	Lys	176	Gly	70	Ala	47	Ala	321	Trp	108	Phe	284	Asp	38
7	Glu	113	His	29	Ile	27	Ala	374	Arg	43	His	71	Tyr	36
8	Asn	96	Val	53	Lys	43	Ile	265	Glu	80	His	65	Gln	45
9	Asp	83	Asn	55	Glu	20	Gln	220	Val	92	Gly	179	Asn	41
10	Ala	122	His	24	Lys	42	Gly	204	Glu	65	Lys	221	Leu	44
11	His	53	Trp	17	Trp	7	Ser	178	Lys	105	His	49	Arg	54
12	Lys	68	Ile	26	Gly	16	Gly	169	Arg	31	His	54	Pro	32
13	Ile	52	Tyr	42	Ser	18	Trp	67	Tyr	90	Gln	104	Lys	30
14	Ala	57	Trp	14			Gly	148	Leu	89	Ala	63	Tyr	23
15	Thr	31	Asp	14			Trp	68	Gln	61	Tyr	66	Leu	23
16	Leu	64	Asn	24			Leu	124	Ala	63	Val	68	Thr	23
17	Gln	46	Leu	35			Gly	94	Ile	51	Asn	59	Glu	7
18	Ser	26	Ala	33			Tyr	112	Glu	19	Asn	63		
19	Ala	43	Pro	19			Asp	68			Leu	56		
20	Leu	50	Val	22			Thr	61			Asn	35		
21	Arg	13	Lys	26			Val	55			Ala	34		
22	Phe	24	Ser	24			Ser	38			Thr	28		
23	Asn	29	Gly	18			Lys	54			Tyr	23		
24	Leu	28	Gly	19			Ser	34			Glu	15		
25			Gly	24			Leu	38						
26			Val	15			Arg	16						
27			Leu	24			Leu	23						
28			Pro	12										
29			Asp	5										
30			Glu	12										
31			His	NQ[a]										
32			Ser	10										
33			Pro	10										
34			Leu	10										
35			Thr	NQ[a]										
36			Lys	NQ[a]										
37			Ala	5										
Repetitive yield %	92.9 (Ala 2-Ala 10)		91.4 (Gly 5-Gly 23)		95.4 (Lys 5-Lys 10)		90.9 (Ile 8-Leu 16)		92.1 (Val 4-Val 9)		94.3 (His 2-His 7)		96.3 (Leu 5-Leu 10)	
Position	39-69		59-99		90-109		110-136		179-196		14-37		161-177	

[a] NQ: not quantitated