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The Primary Structure of the Flavoprotein D-Aspartate Oxidase from Beef Kidney*

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The complete primary structure of the peroxisomal flavoenzyme D-aspartate oxidase from beef kidney has been determined by analyses of the peptides obtained through fragmentation of the carboxymethylated protein with trypsin, CNBr, heptafluorobutyric acid/CNBr and *Staphylococcus aureus* V8 protease. The protein consists of a single polypeptide of 338 residues, accounting for a M_r of 37,305 for the apoprotein. A form of the enzyme lacking Lys-338 and therefore ending with Pro-337 has been detected.

The N-terminal residue is blocked. Seven cysteines and no disulfide bridges are present. Residue 228 can be either Ile or Val. Thus, D-aspartate oxidase presents two types of heterogeneity in the polypeptide chain in addition to the one already described concerning the possible content of FAD or 6-hydroxyflavin adenine dinucleotide.

Comparison of the primary structure of D-aspartate oxidase with other known sequences reveals that D-aspartate oxidase is homologous with D-amino acid oxidase (another flavo-oxidase) and does not present significant sequence similarities with any other protein, including flavoenzymes.

D-Aspartate oxidase (EC 1.4.3.1) is a flavoprotein that catalyzes the oxidative deamination of dicarboxylic D-amino acids by O_2 with the production of H_2O_2, NH_3, and the corresponding α-keto acids (1). Since the discovery of a D-aspartate oxidase activity in tissue homogenates, it became clear that the enzyme responsible for this activity is distinct from the other well known flavoenzyme D-amino acid oxidase (EC 1.4.3.3), which catalyzes the same reaction but is active only toward neutral and basic amino acids.

D-Aspartate oxidase has been so far purified only from the hepatopancreas of *Octopus vulgaris*. (2) and beef kidney cortex (3). The spectrophotometric properties and kinetic mechanism of the beef enzyme have been investigated in detail (3, 4). These studies showed that the general properties of D-aspartate oxidase resemble those of D-amino acid oxidase. The latter is still considered a model for the understanding of the mechanism of action of flavoprotein oxidases. For this reason, we extended the comparison between the two enzymes at the primary structure level by determining the amino acid sequence of beef kidney D-aspartate oxidase and comparing it with the one of D-amino acid oxidase from mammals (5–7), yeast (8), and bacteria (9). This information will be useful in order to understand the evolution of these molecules, in view of a possible explanation for their physiological role, which is still unclear.

This report presents the experimental work that allowed the determination of the amino acid sequence of D-aspartate oxidase.

EXPERIMENTAL PROCEDURES AND RESULTS[1,2]

DISCUSSION

This paper reports for the first time the characterization of the primary structure of the flavoprotein D-aspartate oxidase from beef kidney. The enzyme contains 338 amino acid residues, accounting for a M_r of 37,305 for the apoprotein. This value agrees with the one determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and gel filtration, suggesting that also this peroxisomal enzyme is not a glycoprotein, despite the presence of *N*-glycosylation consensus sequences (Asn-*X*-Ser/Thr) at positions 181–183, 230–232, and 243–245.

The N-terminal residue is blocked. Seven cysteines and no disulfide bridges are present. The primary structure has been determined by sequencing the fragments obtained by digestion of the carboxymethylated enzyme with trypsin, CNBr, CNBr/HFBA, and *Staphylococcus aureus* protease. Experimental evidence for the amino acid sequence shown in Fig. 9 has been obtained for all but the first amino acid residue, which has been indicated as Met on the basis of the following observations: (*a*) the sequence of C2 shows an N-terminal extension of 4 amino acid residues with respect to T2, the first tryptic peptide sequenced; (*b*) the amino acid composition of the tryptic peptide with retention time of 25 min in Fig. 1, whose N terminus is blocked, agrees with the one expected for T1 (Table I); (*c*) the amino acid composition of the protein deduced from the sequence is in good agreement

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) M91559.

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[1] Portions of this paper (including "Experimental Procedures" and "Results," Figs. 1–8, and Tables I and II) 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.

[2] The abbreviations used are: HFBA, heptafluorobutyric acid; PTH, phenylthiohydantoin; GnCl, guanidine hydrochloride; TFA, trifluoroacetic acid; DTNB, 5,5′-dithiobis(2-nitrobenzoic) acid; PVDF, polyvinylidene difluoride; TPCK, *N*-*p*-tosyl-L-phenylalanine chloromethyl ketone.

with the one determined by amino acid analysis (Table I); (*d*) no additional short peptides deriving from fragmentation with CNBr were isolated, suggesting that no other amino acid residues besides a single Met is present before C2. The identification of the C-terminal residue posed some problems due to the lack of information derived from the direct sequence of the entire protein through digestion with carboxypeptidase. In addition, since the last residue is Lys, no tryptic peptide lacking a basic residue at its C terminus was detected, except for T37a, which, however, appears to derive from a form of the enzyme that underwent partial C-terminal proteolysis either in the cell or during the purification procedure of the native enzyme. The assignment of T37 as the C-terminal tryptic peptide is based on the sequence of C7. This CNBr peptide, in fact, overlaps T35, T36, and T37 and lacks a methionine residue at its C terminus. In conclusion, these data show that the enzyme is isolated as a mixture of two forms differing for the C-terminal residue that can be either Pro-337 or Lys-338. This observation is important in view of the role proposed for the C-terminal tripeptide in many peroxisomal enzymes as a "peroxisomal targeting signal" (10). The data presented here for D-aspartate oxidase suggest that such a tripeptide (usually Ser/His(Lys)/Leu) is absent, indicating that some different structural feature fulfills the requirement for the targeting of this enzyme to peroxisomes. However, since Pro has been shown to be a possible substitute for Ser as the first residue of the tripeptide (10), it is also possible that a third residue following Lys-338 (Leu or a residue functionally equivalent for protein targeting) has been proteolytically lost in a maneer similar to what is observed for Lys-338.

Microheterogeneity has also been observed at position 228, which can either be Val or Ile. However, this substitution represents a conservative replacement, originated by a single point mutation in the gene, and is not expected to have a major effect on the general properties of the enzyme. In conclusion, this study reveals that D-aspartate oxidase shows molecular microheterogeneity in the polypeptide chain, in addition to the one already described concerning the possible content of FAD or 6-hydroxyflavin adenine dinucleotide in the isolated protein (3).

Fig. 10 shows the alignment of the amino acid sequence of D-aspartate oxidase from bovine kidney and that of several D-amino acid oxidases from various sources. The "consensus sequence," comprising 137 out of 387 residues (35%) was obtained, considering those amino acids that are identical or conservatively substituted in at least six out of the seven sequences shown. It is interesting to note that D-aspartate oxidase presents 125 of the 137 residues of the consensus sequence (91%), pig, human, and mouse D-amino acid oxidase presents 137 (100%), rabbit D-amino acid oxidase presents 134 (98%), *Trigonopsis variabilis* D-amino acid oxidase presents 107 (78%), and *Fusarium solani* D-amino acid oxidase presents 115 (84%). This observation suggests that indeed D-aspartate oxidase retains most of the primary structural characteristics common to D-amino acid oxidases. Inspection of the consensus sequence reveals that it comprises residues belonging to all the different portions of the molecules: 39, 50, and 48 conserved amino acids are present between residues 1 and 120, 121 and 240, and 241 and 387 of the consensus sequence, respectively. It can be concluded that D-aspartate oxidase and D-amino acid oxidase derive from divergent evolution of a single gene.

When the comparative analysis is restricted to D-aspartate oxidase from beef kidney and D-amino acid oxidase from pig kidney (the usual and best studied source of this enzyme), the

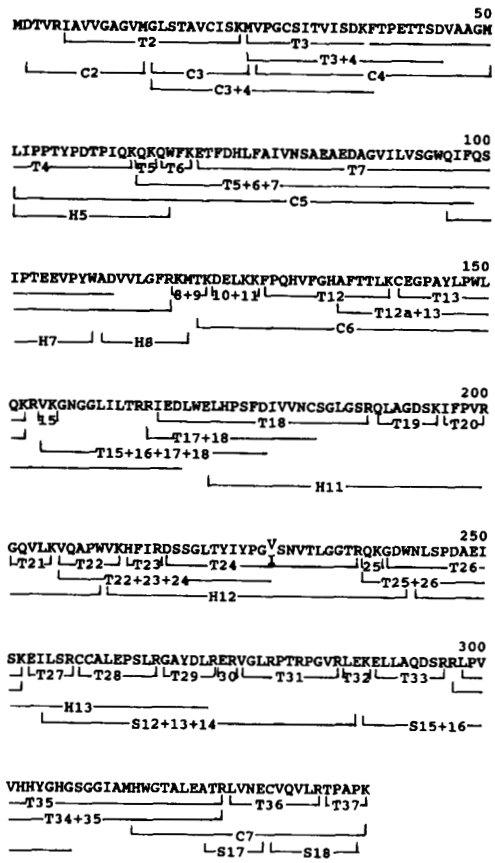

FIG. 9. **The complete amino acid sequence of beef kidney D-aspartate oxidase.** *T*, tryptic peptides; *C*, CNBr peptides; *H*, CNBr/HFBA peptides; *S*, *S. aureus* V8 peptides.

two proteins appear to be homologous (41% identities and an additional 15% of conservative replacements). In addition, the observation that 75% of the glycines and tryptophans and 60% of the prolines are conserved suggests also that the tertiary structure of the two enzymes must be very similar.

The role of active site residues in pig kidney D-amino acid oxidase has been extensively investigated using both chemical modification and site-directed mutagenesis techniques (7, 11). According to the alignment shown in Fig. 10, Tyr-228 and His-307 (the numbers refer to pig kidney D-amino acid oxidase) are punctually conserved in all the enzymes, suggesting a common catalytic role for these residues that, however, has not yet been elucidated. None of the other "active site residues" (11) (Tyr-55, Met-110, Lys-204, His-217, and Tyr-224; marked with an *asterisk* in Fig. 10) are conserved in all the proteins considered, although in most cases, functionally equivalent residues can be found around the position of those amino acids in the sequence of the enzymes.

No other proteins besides those shown in Fig. 10 present extended primary structure similarity with D-aspartate oxidase, except for the N-terminal sequence, which is known to be common to most enzymes binding ADP-containing nucleotides (12). In particular, no significant similarities were detected between D-aspartate oxidase and the FMN- containing flavoproteins of the oxidase/dehydrogenase class that have been shown to share a common mechanism of catalysis with D-amino acid oxidase (13) (lactate oxidase, glycolate oxidase, and flavocytochrome b_2 (14–16)). This observation reinforces the proposal of Geigel *et al.* (14) that D-aspartate oxidase and D-amino acid oxidase (both FAD-containing enzymes) constitute a family of homologous proteins different from the one comprising the FMN-containing oxidases and

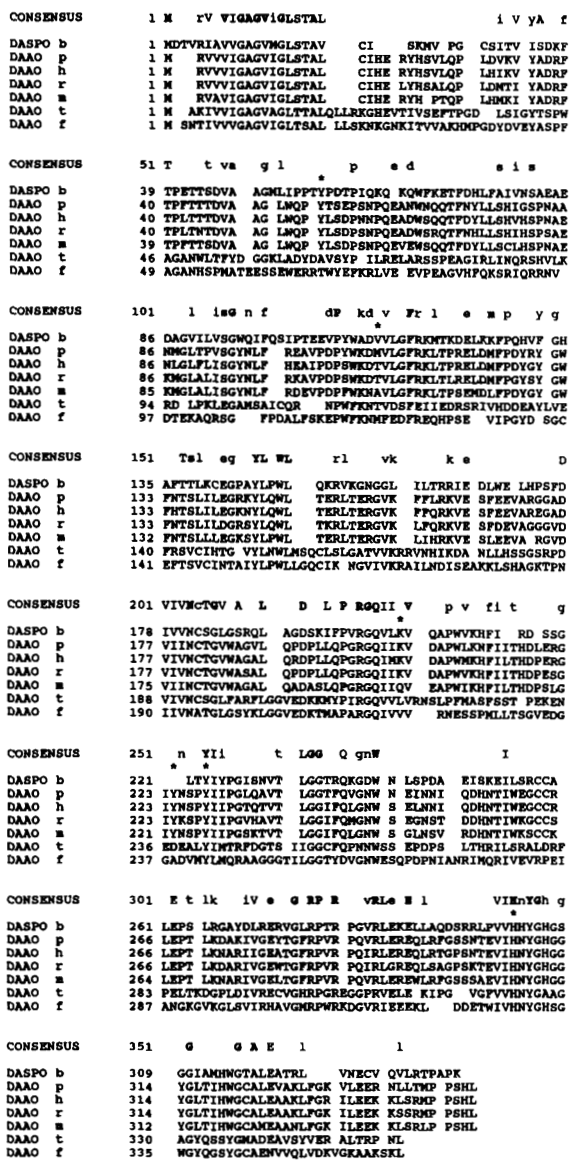

FIG. 10. **Comparison of the primary structure of D-aspartate oxidase with that of D-amino acid oxidase from various sources.** *DASPO b*, bovine D-aspartate oxidase; *DAAO p*, pig D-amino acid oxidase (5); *DAAO h*, human D-amino acid oxidase (6); *DAAO r*, rabbit D-amino acid oxidase (7); *DAAO m*, mouse D-amino acid oxidase (7); *DAAO t*, *T. variabilis* D-amino acid oxidase (8); *DAAO f*, *F. solani* D-amino acid oxidase (9). The consensus sequence was determined, considering the residues that were identical in all seven sequences (*bold upper case*), identical or conservatively replaced in all seven sequences (*upper case*), and identical or conservatively replaced in at least six out of seven sequences (*lower case*). *Asterisks* mark the active site residues of mammalian D-amino acid oxidase proposed on the basis of chemical modification and site-directed mutagenesis studies (7, 11).

that the two families evolved toward a common chemical mechanism of catalysis through convergent evolution.

No extended sequence similarities were found between D-aspartate oxidase, L-aspartate oxidase from *Escherichia coli*, and L-amino acid oxidase from *Neurospora crassa*. The latter two enzymes are FAD-containing oxidases constituted by 540 and 566 amino acids, respectively (17, 18). The different masses and the absence of sequence similarities between the oxidases active on D- or L-amino acids, which result in the completely different substrate specificity exerted by the two groups of enzymes, probably derive from the need to mantain a strict control on the entrance of D- or L-amino acids in different metabolic pathways. The growing number of information concerning the presence and the possible biological role of D-amino acids will permit a conclusion whether D-aspartate oxidase and D-amino acid oxidase are still to be considered "detoxifying enzymes" (19) or are involved in more complex metabolic pathways.

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Supplemental Material to

THE PRIMARY STRUCTURE OF THE FLAVOPROTEIN D-ASPARTATE OXIDASE

FROM BEEF KIDNEY

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EXPERIMENTAL PROCEDURE

Materials. Beef kidney D-aspartate oxidase was purified as described in (3). TFA, 6 N HCl, HFBA, CNBr and GnCl were from Pierce Chemical Company. TPCK-trypsin, *Staphylococcus aureus* protease and carboxypeptidase B were obtained from Boehringer Mannheim. Iodo[2-^{14}C]acetic acid was from Amersham. All the other reagents used were of the highest purity commercially available.

Amino acid analysis. Gas-phase hydrolysis was carried out in 6 N HCl containing 1 % (v/v) phenol at 105 °C for 24 h. Amino acid analyses were carried out by a post-column o-phtalhaldeyde derivatization procedure (20), using a Jasco (Japan Spectroscopy) amino acid analyzer equipped with a 880-PU pump and a 820-FP detector. Cysteine content was determined either as carboxymethylcysteine after reduction and carboxymethylation of the protein or by titration with DTNB under denaturing non riducing conditions in 50 mM KPi, 6 M GnCl, pH 7.4 (21).

Carboxymethylation. Reduction and carboxymethylation of the protein were performed as described in (22). The material to be subjected to tryptic digestion was derivatized using iodo[2-^{14}C]acetic acid 1 μCi / μmol.

Enzymatic cleavages. Tryptic digestion was carried out in 0.1 % NH_4HCO_3, pH 8.0 at 37 °C for 4 h using a protease : carboxymethylated D-aspartate oxidase ratio of 1 : 100. A second aliquot of protease was then added and the digestion was allowed to proceed for an additional hour under the same conditions. The reaction was stopped by lowering the pH below 2.0 with TFA followed by lyophylization. The material was then resuspended in 500 μl of 0.1 % TFA and centrifuged for 8 min using an Eppendorf 5414 centrifuge. The pellet was washed with 100 μl of 0.1 % TFA and centrifuged as above. The supernatants from the two centrifugations were pooled and the peptides (0.1 % TFA soluble peptides) chromatographed on a RP-HPLC column as described below. The pellet (0.1 % TFA insoluble material) was dissolved in 400 μl of 30 % CH_3COOH, centrifuged and separated on a second RP-HPLC chromatography. No insoluble material was present after solubilization with 30 % CH_3COOH.

Digestion with *S. aureus* protease was carried out for 24 h at 37 °C using a protease : carboxymethylated D-aspartate oxidase ratio of 1 : 25 (w /w), in 50 mM ammonium acetate, pH 4.0.

Carboxypeptidase B digestion was conducted both on the carboxymethylated enzyme (using a protease : substrate ratio of 1 : 25 (w/w) in 0.2 M N-ethylmorpholine acetate, 1 M urea, pH 8.5. Aliquots of the incubation mixture were withdrawn at different times, the proteins precipitated with 10 % trichloroacetic acid and centrifuged, and the supernatant analyzed for amino acid content.

Chemical cleavages. Cleavage at methionyl residues was accomplished by dissolving 0.5 mg of carboxymethylated protein in 70 % formic acid and adding a 100-fold molar excess of CNBr over methionyl residues. After 24 h at room temperature in the dark the reaction was stopped by a 10-fold dilution with water followed by liophylization.

Further cleavage of the CNBr peptides at tryptophanyl residue was carried out by dissolving the peptides in 2 ml of 70 % formic acid : HFBA 1 : 1 and adding a 10,000-fold molar excess of solid CNBr over tryptophanyl residues (23). After 24 h at room temperature the mixture was dried with a stream of nitrogen in hood, dissolved in 10 ml of water and liophylized.

Reverse-phase HPLC chromatography. Purification of peptides was carried out by RP-HPLC using a JASCO (Japan Spectroscopy) HPLC equipped with a n Uvidec-100 V detector set at 220 nm and an Aquapore C-8 RP-300 (4.6 x 25 mm) column (Applied Biosystems). The chromatographic conditions used are indicated in the Figure legends. Aliquots from each fraction were withdrawn and analyzed for amino acid content, [^{14}C] incorporation and amino acid sequence.

[^{14}C] incorporation measurement. [^{14}C] incorporation was measured by dissolving aliquots of [^{14}C]-carboxymethylated enzyme, HPLC- purified peptides or PTH-amino acid derivatives originated during the Edman degradation and recovered from the sequencer in Instagel (Packard) and counting in a Packard Minaxi Tricarb liquid scintillation counter.

Sequence analysis. Sequence analyses were carried out on an Applied Biosystems Model 477-A pulse liquid-phase sequencer equipped with a Model 120-A PTH-analyzer according to the manufecturer's instructions. Identification of PTH-[^{14}C]- carboxymethylcysteine was confirmed by [^{14}C] incorporation measurements. Blotting of the protein on PVDF membrane prior to sequencing was performed according to (24).

Computer analysis. A search for similarities between the primary structure of D-aspartate oxidase with the one of other proteins was performed using the Swiss-Prot Protein Database.

RESULTS

The N-terminal residue of D-aspartate oxidase is blocked, as indicated by the fact that no sequence informations were obtained by submitting either the native, carboxymethylated or PVDF- membrane blotted protein to Edman degradation. Thus, the determination of the amino acid sequence of D-aspartate oxidase was accomplished through the characterization of all the peptides obtained by tryptic digestion of the carboxymethylated protein. Alignment of the tryptic peptides was subsequentally achieved by submitting the carboxymethylated protein to different enzymatic or chemical fragmentations.

Tryptic digest. Tryptic digestion of 3 mg D-aspartate oxidase gave two sets of peptides: (a) soluble in 0.1 % TFA and (b) insoluble in 0.1% TFA. The two sets were separated by centrifugation as indicated under "Experimental Procedure". Peptides contained in set (a) were separated directly by RP-HPLC (Fig. 1). The peptides co-eluted in the fractions with retention time of 32, 35 and 43 min were further purified by RP-HPLC under different conditions (Figs. 2, 3 and 4). The 0.1% TFA insoluble material was first solubilized in 30% CH_3COOH and then separated by RP-HPLC (Fig. 5). Fractions from each chromatography were analyzed for amino acid composition, [^{14}C]-incorporation and amino acid sequence. Tables I and II and Figure 9 report the results of such analyses for all the tryptic peptides characterized. Since the digestion conditions (i.e. protease to substrate ratio and time of digestion) were relatively mild a significant number of partially digested peptides were obtained. This, however, allowed immediate alignment of many short peptides, as shown in Figure 9. No amino acid sequence was obtained by submitting the peptide with retention time 25 min of Figure 1 to Edman degradation. This peptide was assigned as T1 since its N-terminus is blocked, as expected from the analysis of the entire protein, and its amino acid composition (Table I) corresponds to the one expected for this portion of the molecule on the basis of the results obtained through the analysis of the CNBr fragments (see below).

In conclusion, the characterization of the tryptic peptides allowed to estabilish portions of the sequence accounting for 333 out of the total 338 amino acid residues which constitute the enzyme. Complete alignment of the tryptic peptides and identification of the sequence of the 5 amino acid residues still missing (positions 1-5) were achieved by the characterization of the peptides obtained by fragmentation of the carboxymethylated protein with CNBr, CNBr/HFBA and *S. aureus* V8 protease.

CNBr and CNBr/HFBA digests. RP-HPLC separation of the petides obtained by fragmentation of the carboxymethylated protein with CNBr is shown in Figure 6. The number of peaks largely exceeded the number of peptides expected from the methionine content as determined by amino acid analysis of the protein (Table I). Characterization of all the HPLC fractions revealed that some peaks contained the same peptide, sometimes in various mixtures with others, or partially fragmented portions. However, C2, C3, C4 and C7 were obtained as pure peptides and their sequence was unambigously determined (Table II and Fig. 9). The large fragments C5 and C6 instead were isolated only as a mixture. Sequence analysis performed on aliquots of the fractions eluting between 80 and 86 min and containing C5 and C6 resulted in more than one amino acid released for each degradation cycle. In this case, tentative assignment of the amino acid sequence for each fragment was deduced by comparison with the known primary sequence of the corresponding tryptic peptides previously characterized. The remaining material from the HPLC fractions containing C5 and C6 was pooled and further fragmented at tryptophanyl residues as described under "Experimental Conditions". RP-HPLC separation of the peptides obtained is shown in Figure 7. Again, only the data obtained from the analysis of the pure peptides have been used to determine the alignment described in Figure 9.

Staphylococcus aureus V8 digest. Experimental evidence for the alignment of the tryptic peptides comprising the C-terminal portion of the molecule (T27-T37) was obtained by the characterization of a few peptides originated by digesting the protein with *S.aureus* V8 protease (Fig. 8). Both native and carboxymethylated D-aspartate oxidase resulted to be very resistant to digestion. In fact, only the C- terminal portion of the molecule (starting after Glu253) appeared to be sensitive to the action of the protease.

Special problems. Apart from the failure in sequencing the N- terminal portion of the entire protein , the unambigous assignment of a few other portions of the molecule required special attention. In particular, no C-terminal sequence was obtained by submitting the carboxymethylated protein to digestion with carboxypeptidase. Thus, the C-terminus of the protein has been identified as Lys338 from the amino acid sequence of T37 and CB7. However the sequence of tryptic peptide T37a is equal to the one of T37 except for the absence of the terminal Lys residue. Determination of the amino acid composition of T37 and T37a confims that these peptides differ only for one Lys residue (Table I). In addition, S18 also ends with Pro337. Taken together these results suggest that partial digestion of the C-terminal residue might occur, either in the cell or during the purification of the native protein, originating two forms of the enzyme ending with Lys338 or with Pro337.

Another type of molecular microheterogeinity has been detected, deriving from genetic variants of the enzyme. In fact, either Val or Ile were found at position 228 (peptide T24 and T24a, respectively). Finally, overlapping of the tryptic peptides was in some cases complicated by a few peculiar "tryptic" cleavages, such as between His134 and Ala135, Leu160 and Ile 161 and Asn181 and Cys182 (Fig. 9).

Table II and Figure 9 summarize the data used to determine the complete primary structure of beef kidney D-aspartate oxidase. The enzyme consists of 338 amino acid residues, accounting for a M_r of 37305 for the apoprotein, in accordance with the value reported in previous studies (3). Table I shows the good correlation between the amino acid composition of the entire protein determined by amino acid analysis with the one deduced from the sequence. The data relative to the Cys content suggest that the enzyme contains 7 Cys and no disulfide bridges, since all the Cys can be titrated by DTNB under denaturing non-reducing conditions.

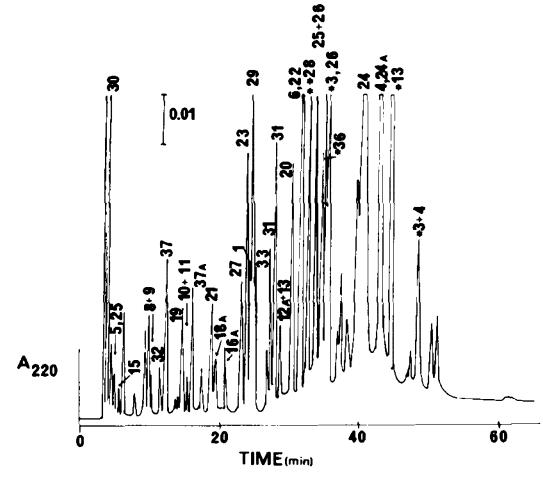

Fig 1. RP-HPLC of the 0.1 % TFA-soluble peptides obtained by tryptic digestion of [^{14}C]-carboxymethylathed D-aspartate oxidase. Conditions: column C-8 Aquapore RP-300 (250 x 7 mm); buffer A: 0.1 % TFA; buffer B: CH_3CN + 0.075 % TFA; flow rate 2 ml/min. Gradient: from 0 to 60% B in 85 min. Fraction numbers refer to the tryptic peptides found in each peak and aligned as shown in Fig. 9. An asterisk marks peptides containing [^{14}C]-carboxymethylcysteine determined by radioactivity counting both on an aliquot of each fraction and of the PTH-derivative recovered from the Edman degradation. The peptides contained in fractions with retention time of 32, 35 and 43 min were identified after re-purification as described in Fig. 3, 4 and 5, respectively.

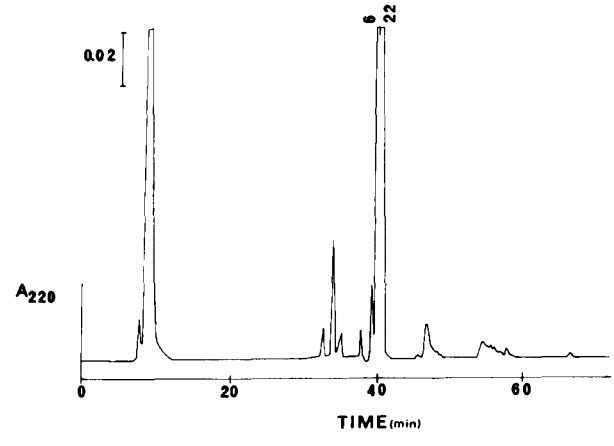

Fig. 2. RP-HPLC purification of peptides contained in fraction with retention time of 32 min in Fig.1. Conditions: column C-8 Aquapore RP- 300 (250 x 4.6 mm), flow rate 0.7 ml/min; buffer A: 5 mM $NaPi_i$ pH 6.0; buffer B: CH_3CN. Gradient: from 0 to 60 % B in 85 min.

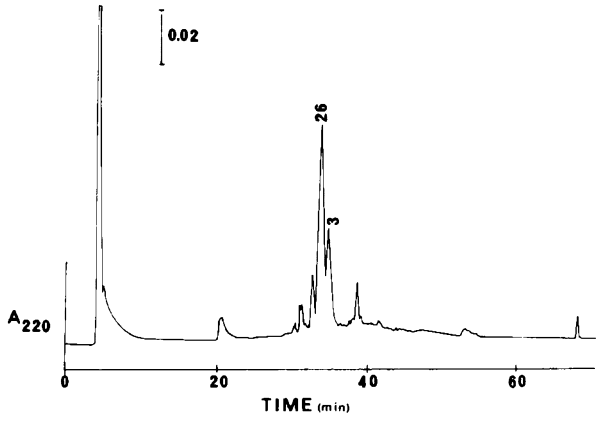

Fig. 3. RP-HPLC purification of peptides contained in fraction with retention time of 35 min in Fig. 1. Conditions as in Fig. 3.

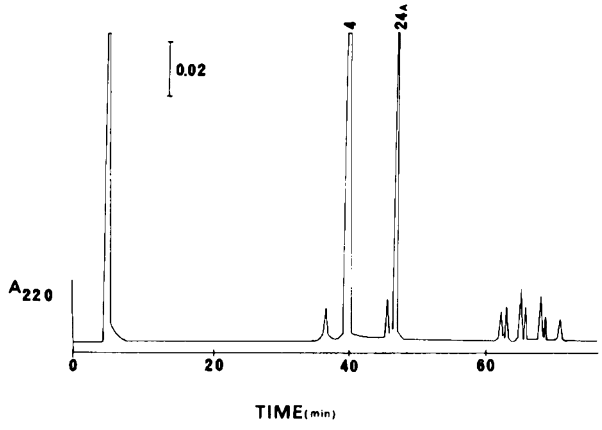

Fig. 4. RP-HPLC purification of peptides contained in fraction with retention time of 43 min in Fig. 1. Conditions as in Fig. 3.

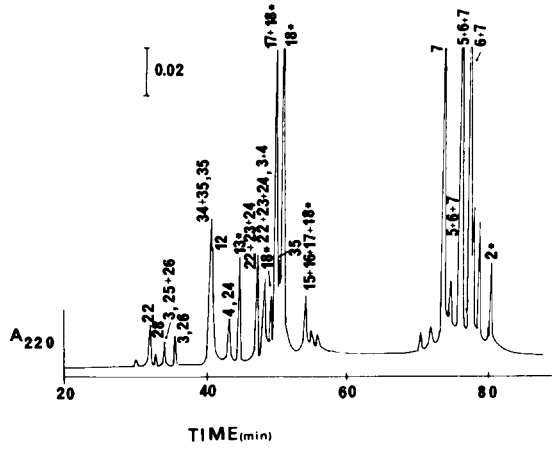

Fig. 5. RP-HPLC of the 0.1 % TFA-insoluble peptides obtained by tryptic digestion of [^{14}C]-carboxymethylathed D-aspartate oxidase. Conditions as in Fig. 1.

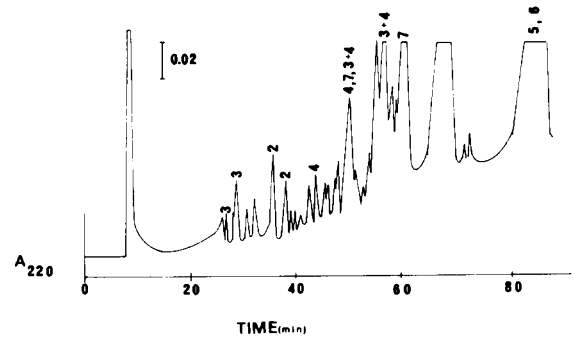

Fig. 6. RP-HPLC of peptides obtained by CNBr fragmentation of S- carboxymethylated D-aspartate oxidase. Conditions: column C-8 Aquapore RP-300 (250 x 4.6 mm); flow rate 0.7 ml/min; buffer A: 0.1 % TFA; buffer B: CH_3CN + 0.075 % TFA. Gradient: from 0 to 60% B in 85 min.

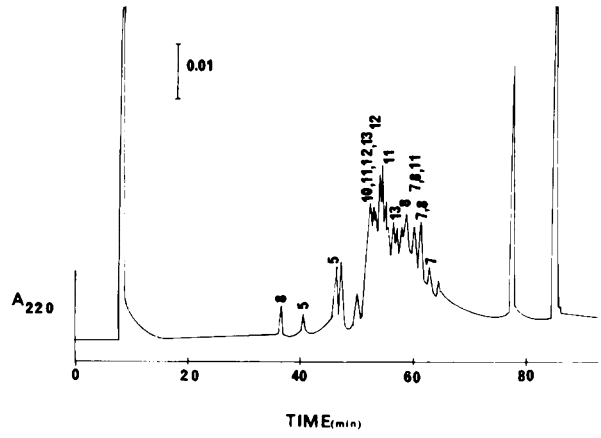

Fig. 7. RP-HPLC of peptides obtained by CNBr/HFBA fragmentation of peptides C5 and C6 contained in fractions with retention time ranging from 80 to 85 min in Fig. 6. Conditions as in Fig. 6.

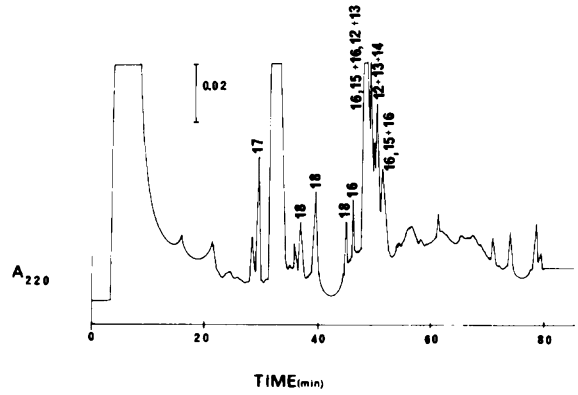

Fig. 8. RP-HPLC of peptides obtained by digestion with S. aureus protease of S-carboxymethylated D-aspartate oxidase. Conditions as in Fig. 6.

Table I

Amino acid composition of the entire protein and of the peptides generated by tryptic digestion of S-carboxymethylated enzyme

AA	Whole	T1	T2	T3	T4	T5	T6
Cys[a]	7.0(7)		0.6(1)	0.8(1)			
Asx	23.0(22)	1.0(1)		1.1(1)	2.3(2)		
Thr	20.9(21)	0.7(1)	0.8(1)	0.6(1)	4.6(5)		
Ser	20.4(21)		1.7(2)	1.6(2)	1.6(1)		
Glx	30.5(31)				2.3(2)	0.9(1)	1.0(1)
Pro	22.0(22)			0.9(1)	3.9(5)		
Gly	29.0(30)		3.2(3)	0.9(1)	1.1(1)		
Ala	24.3(22)		3.0(3)		1.9(2)		
Val	27.2(30)	0.6(1)	3.3(4)	1.8(2)	1.1(1)		
Met	5.4(6)	1.2(1)	0.7(1)	0.5(1)	1.0(1)		
Ile	18.0(20)		1.6(2)	1.5(2)	2.1(2)		
Leu	32.6(31)		1.0(1)		1.1(1)		
Tyr	6.6(7)				1.0(1)		
Phe	11.8(12)				1.0(1)		0.9(1)
Lys	19.4(19)		1.0(1)	1.0(1)	1.0(1)	1.1(1)	1.0(1)
His	8.7(9)						
Arg	20.0(20)	1.0(1)					
Trp[b]	8.8(8)						n.d(1)
Pos	1-338	1-5	6-24	25-37	38-63	64-65	66-69
(%)		8	15	20	51	23	22

AA	T6+7	T8+9	T10+11	T12	T13	T15	T15+16+17+18
Cys					0.9(1)		0.6(1)
Asx	3.8(4)		1.2(1)				4.2(4)
Thr	1.4(2)	1.0(1)		2.1(2)			0.5(1)
Ser	2.4(3)						2.7(3)
Glx	6.9(8)		1.1(1)	1.1(1)	2.1(2)		1.7(2)
Pro	2.2(2)			0.9(1)	2.4(2)		1.4(1)
Gly	2.9(3)			1.3(1)	1.0(1)		4.6(5)
Ala	4.7(5)			1.1(1)	0.7(1)		
Val	5.3(6)			0.7(1)		1.0(1)	2.4(3)
Met		0.6(1)					
Ile	3.2(4)						3.5(3)
Leu	3.1(3)		1.0(1)	1.0(1)	1.8(2)		5.5(5)
Tyr	0.4(1)				0.7(1)		
Phe	4.4(5)			2.8(3)			1.0(1)
Lys	1.0(1)	2.0(2)	2.0(2)	0.9(1)	1.0(1)	0.9(1)	1.2(1)
His	0.7(1)			1.5(2)			0.8(1)
Arg	0.9(1)						2.6(3)
Trp	n.d(1)				n.d(1)		n.d(1)
Pos	66-117	118-121	122-126	127-140	141-152	154-155	154-188
(%)	15	9	11	9	87	10	6

Table I (continued)

AA	T17+18	T18	T19	T20	T21	T22	T23
Cys	0.7(1)	0.6(1)					
Asx	2.8(3)	2.9(3)	1.0(1)				
Thr							
Ser	1.9(3)	2.0(3)	0.6(1)				
Glx	2.1(2)	2.1(2)	1.2(1)		1.0(1)	1.4(1)	
Pro	1.0(1)	1.1(1)		0.8(1)		1.1(1)	
Gly	2.4(2)	2.0(2)	0.9(1)		1.3(1)		
Ala			0.7(1)			1.1(1)	
Val	1.7(2)	1.7(2)		0.4(1)	0.8(1)	1.9(2)	
Met							
Ile	1.4(2)	1.5(2)		0.6(1)			0.8(1)
Leu	3.2(3)	3.1(3)	1.0(1)		1.1(1)		
Tyr							
Phe	1.1(1)	0.9(1)		1.0(1)			1.0(1)
Lys			1.1(1)		1.0(1)	1.0(1)	
His	1.0(1)	1.0(1)					0.6(1)
Arg	2.0(2)	1.0(1)		1.0(1)			1.0(1)
Trp	n.d(1)	n.d(1)				n.d(1)	
Pos	165-188	166-188	189-195	196-200	201-205	206-212	213-216
(%)	23	31	73	77	86	53	67

AA	T24a	T24	T25	T26	T27	T28	T29
Cys						1.8(2)	
Asx	2.1(2)	2.2(2)		3.0(3)			1.2(1)
Thr	3.2(3)	3.3(3)					
Ser	2.5(3)	2.7(3)		1.7(2)	1.1(1)	0.9(1)	
Glx			0.8(1)	1.2(1)	1.3(1)	1.2(1)	
Pro	0.7(1)	0.8(1)		0.7(1)		2.1(1)	
Gly	4.1(4)	4.4(4)		0.8(1)			0.9(1)
Ala				1.0(1)		1.2(1)	0.9(1)
Val	0.8(1)	1.9(2)					
Met							
Ile	1.9(2)	0.8(1)		0.9(1)	0.6(1)		
Leu	2.0(2)	2.0(2)		1.0(1)	1.0(1)	2.4(2)	1.2(1)
Tyr	2.0(2)	1.5(2)					0.5(1)
Phe							
Lys			1.0(1)	1.0(1)			
His							
Arg	1.0(1)	1.0(1)			1.0(1)	1.0(1)	1.0(1)
Trp				n.d(1)			
Pos	217-237	217-237	238-239	240-252	253-257	258-266	267-272
(%)	24	29	23	14	126	89	105

Table I (continued)

AA	T30	T31	T32	T33	T34+35	T36	T37	T37a
Cys						0.7(1)		
Asx				1.0(1)		1.2(1)		
Thr		0.8(1)			1.6(2)		0.9(1)	0.9(1)
Ser				0.6(1)	0.8(1)			
Glx	1.0(1)		1.0(1)	2.0(2)	1.2(1)	1.8(2)		
Pro		1.4(2)			1.0(1)		2.1(2)	1.9(2)
Gly		2.1(2)			5.2(5)			
Ala				0.7(1)	1.4(3)		1.1(1)	1.1(1)
Val		1.6(2)			1.6(2)	3.3(3)		
Met					0.7(1)			
Ile					0.8(1)			
Leu		1.2(1)	1.1(1)	2.0(2)	2.0(2)	1.8(2)		
Tyr					0.8(1)			
Phe								
Lys			0.9(1)				1.1(1)	0.1(0)
His					3.4(4)			
Arg	1.0(1)	3.0(3)		1.0(1)	1.6(2)	1.0(1)		
Trp					n.d(1)			
Pos	273-274	275-285	286-288	298-296	297-323	324-333	334-338	334-337
(%)	45	58	42	100	30	86	46	11

Footnotes to Table I. [a]Cys content of the entire protein was determined both by amino acid analysis as S-carboxymethylcysteine and by DTNB titration. [b]Determined by second derivative spectroscopy (From Ref. 3). Pos indicates the position of the peptide in the primary sequence. (%) Indicates the recovery yield of each peptide.

Table II

Amino acid sequence of peptides obtained through various chemical or enzymatic fragmentation of [^{14}C]-carboxymethylated D-aspartate oxidase.

1.M														
2.D												C2	994	C2 77
3.T													1426	50
4.V													2958	199
5.R													165	20
6.I	T2	32											2089	211
7.A		25											3147	117
8.V		21											2853	170
9.V		38											3000	184
10.G		38											3051	135
11.A		23											2969	72
12.G		36											1908	129
13.V		21											464	26
14.M		6											hsl	-
15.G		34						C3+4	138			C3	313	C3 1198
16.L		14							82				109	1376
17.S		4							19				51	118
18.T		4							28				74	256
19.A		13							53				95	592
20.V		17							55				137	630
21.C		-							20				21	134
22.I		18							57				121	568
23.S		3							11				26	78
24.K		1							29				13	245
25.M	T3	1109							12				hsl	-
26.V		828					T3+4	305	36					C4 308
27.P		293						312	36					149
28.G		1350						194	52					337
29.C		390						262	16					55
30.S		270						138	6					35
31.I		731						89	24					209
32.T		330						206	15					58
33.V		680						93	27					192
34.I		598						94	17					170
35.S		258						158	5					26
36.D		356						46	21					59
37.K		381						56	11					74
38.F	T4	1447						81	11					89
39.T		516						76	-					45
40.P		1362						32						63
41.E		1173						65						14
42.T		431						41						15
43.T		475						22						15
44.S		335						27						12
45.D		672						17						24
46.V		874						19						38
47.A		1093						-						32
48.A		937												52
49.G		608												51
50.M		361												hsl
51.L		235								H5	365		C5* 1440	
52.I		196									325		1383	
53.P		185									210		840	
54.P		167									179		621	
55.T		111									44		307	
56.Y		82									d		532	
57.P		95									91		474	
58.D		68									71		395	
59.T		36									23		128	
60.P		27									55		470	
61.I		34									64		328	
62.Q		33									37		263	
63.K		14									29		258	
64.Q	T5+	491	T5	1001							63		337	
65.K	6+7	279		281							48		420	
66.Q		431	T6	615							86		381	
67.W		107		512							-		251	
68.F		664		422									335	
69.K		763		405									259	
70.E		720	T7	259									140	
71.T		314		120									51	
72.F		434		353									219	
73.D		331		230									84	
74.H		85		36									21	
75.L		219		161									163	
76.F		255		191									155	
77.A		449		244									213	
78.I		228		192									166	
79.V		276		147									207	
80.N		269		202									47	
81.S		106		84									23	
82.A		375		145									131	
83.E		157		105									55	
84.A		338		157									126	
85.E		166		120									51	
86.D		223		104									105	
87.A		237		116									117	
88.G		188		116									288	
89.V		102		42									145	
90.I		72		55									126	
91.L		89		73									307	
92.V		67		28									113	
93.S		34		28									36	
94.G		107		73									236	
95.M		15		5									41	
96.Q		39		45						H7	411		66	
97.I		39		20							423		131	
98.F		43		18							365		69	
99.Q		51		51							374		-	
100.S		19		9							45			
101.I		30		11							294			
102.P		15		17							138			
103.T		11		9							31			
104.E		32		15							55			

105.E	32	20			82	
106.V	18	3			105	
107.P	8	14			59	
108.Y	8	1			d	
109.W	-	2			-	
110.A	18	7		H8 780		
111.D	11	10		578		
112.V	14	-		787		
113.V	17			812		
114.L	10			902		
115.G	9			536		
116.F	8			537		
117.R	-			41		
118.K	T8+9 930			549		
119.M	1189			hsl		
120.T	271					C6* 153
121.K	415					464
122.D	T10+ 608					327
123.E	11 870					287
124.L	2689					704
125.K	975					502
126.K	603					613
127.F	T12 129					463
128.P	60					461
129.Q	73					335
130.H	14					54
131.V	39					168
132.F	78					328
133.G	78					382
134.H	19					53
135.A	69	T12a+13 1210				240
136.F	57	779				103
137.T	27	263				52
138.T	32	281				54
139.L	60	271				249
140.K	10	190				234
141.C	T13 257	320				28
142.E	158	195				122
143.G	323	312				226
144.P	317	170				180
145.A	215	233				207
146.Y	175	141				89
147.L	158	119				203
148.P	151	78				163
149.W	44	13				40
150.L	69	33				186
151.Q	59	43				90
152.K	11	7				97
153.R						23
154.V		T15+16+17+18 189				200
155.K		78				137
156.G		165				211
157.N		108				87
158.G		150				134
159.G		144				169
160.L		92				147
161.I	T16a 96	93				158
162.L	67	83				120
163.T	30	42				31
164.R	13	22				-
165.R		28	T17+18 80			-
166.I		64	191	T18 398		131
167.E		41	95	162		49
168.D		32	78	113		65
169.L		36	107	331		-
170.W		5	67	220		
171.E		22	60	119	H11 180	
172.L		26	123	227	550	
173.H		4	13	15	73	
174.P		26	90	207	250	
175.S		9	27	79	46	
176.F		13	48	208	161	
177.D		15	38	65	129	
178.I		-	43	175	240	
179.V			23	91	139	
180.V			21	87	187	
181.N			30	64	71	
182.C		T18a 653	17	36	52	
183.S		696	-	34	29	
184.G		1067		62	133	
185.L		1464		59	124	
186.G		1472		53	124	
187.S		438		21	26	
188.R		79		5	9	
189.Q	T19 1212				66	
190.L	1270				198	
191.A	1351				66	
192.G	1231				88	
193.D	766				38	
194.S	220				11	
195.K	976				26	
196.I	T20 568				36	
197.F	486				33	
198.P	393				26	
199.V	257				19	
200.R	44				5	
201.G	T21 1660				56	
202.Q	1178				23	
203.V	1233				18	
204.L	1399				41	
205.K	636				16	
206.V		T22 1940	T22+23+24 101		16	
207.Q		1198	128		20	
208.A		1829	126		17	
209.P		1017	83		15	
210.W		738	26		-	
211.V		618	35			H12 246
212.K		520	31			213
213.H	T23 58		13			73
214.F	578		47			157

215.I	617			53		175
216.R	33			28		19
217.D	T24a 1312	T24 779		37		59
218.S	461	375		20		31
219.S	410	388		23		36
220.G	2032	911		44		322
221.L	1423	622		27		351
222.T	486	309		20		42
223.Y	1393	588		12		d
224.I	961	324		19		89
225.Y	1508	490		9		d
226.P	1060	395		22		64
227.G	1817	425		37		51
228.V	945(I)	186		6		48
229.S	214	92		-		16
230.N	590	190				26
231.V	676	105				48
232.T	221	40				22
233.L	739	163				62
234.G	1052	164				75
235.G	1073	139				84
236.T	206	40				14
237.R	46	25				6
238.Q	T25 1001	T25+26 408				
239.K	281	685				
240.G	T26 1760	539				
241.D	1657	453				
242.W	1078	47				
243.N	1261	287		H13 80		
244.L	1280	339		150		
245.S	510	79		29		
246.P	973	166		120		
247.D	1124	119		83		
248.A	1200	119		74		
249.E	998	52		41		
250.I	474	49		90		
251.S	120	33		36		
252.K	160	12		36		
253.E	T27 1290			26		
254.I	1428			85	S12+13+14 74	
255.L	1600			95	60	
256.S	456			32	26	
257.R	237			16	11	
258.C	T28 820			-	21	
259.C	839			-	20	
260.A	1818			23	37	
261.L	969			47	26	
262.E	887			10	19	
263.P	1135			19	18	
264.S	229			12	11	
265.L	724			62	17	
266.R	85			-	6	
267.G	T29 487			24	24	
268.A	852			16	15	
269.Y	969			d	10	
270.D	631			8	7	
271.L	632			14	12	
272.R	114			-	2	
273.E	T30 412				6	
274.R	42				2	
275.V	T31 1535				5	
276.G	1663				15	
277.L	1576				9	
278.R	1202				-	
279.P	1300				8	
280.T	2637				3	
281.R	977				-	
282.P	1164				6	
283.G	1110				10	
284.V	957				7	
285.R	500				-	
286.L	T32 1605				4	
287.E	1376				1	
288.K	1615				S15+16 20	
289.E	T33 1515				21	
290.L	1541				38	
291.L	1055				36	
292.A	1929				50	
293.Q	1126				55	
294.D	985				50	
295.S	479				43	
296.R	531				15	
297.R		T34+35 44			12	
298.L		123	T35 161		22	
299.P		567	89		25	
300.V		827	85		9	
301.V		979	230		18	
302.H		113	35		3	
303.H		155	72		4	
304.Y		486	172		7	
305.G		481	184		12	
306.H		106	55		-	
307.G		514	202		7	
308.S		138	76		-	
309.G		558	193			
310.G		469	221			
311.I		276	62			
312.A		291	32			
313.M		363	48			
314.H		54	20			C7 58
315.W		130	29			207
316.G		191	86			350
317.T		49	38			178
318.A		173	38			321
319.L		248	31			368
320.E		102	20			79
321.A		131	46	S17 91		210
322.T		87	37	45		119
323.R		18	12	30		24
324.L	T36 1371			67		224
325.V	1503			55		326
326.N	979			51		85
327.E	768			15		43
328.C	763			S18 100		36
329.V	1052			215		196
330.Q	805			201		122
331.V	850			152		217
332.L	724			162		179
333.R	103			20		18
334.T	T37 310	T37a 94		47		42
335.P	634	200		65		69
336.A	720	212		33		121
337.P	356	40		15		75
338.K	104			-		12

Footnotes to Table II. Values are given in pmoles. T, C, H and S indicates peptide obtained by fragmentation with trypsin, CNBr, CNBr/HFBA ans *S.aureus* V8 protease, respectively; hsl, indicates homoserine and homoserine lactone; d, indicates a PTH-derivative of tyrosine eluting after PTH-leucine under the experimental condition used for PTH-amino acid separation; * indicates that peptides C5 and C6 were sequenced as a mixture.