Cathepsin D Isozymes from Porcine Spleens

LARGE SCALE PURIFICATION AND POLYPEPTIDE CHAIN ARRANGEMENTS*

Jung San Huang, Shuan Shian Huang, and Jordan Tang

From the Laboratory of Protein Studies, Oklahoma Medical Research Foundation, and the Department of Biochemistry and Molecular Biology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73104

Six cathepsin D isozymes have been purified from porcine spleen using a large scale purification procedure. Five isozymes, I to V, have an identical molecular weight of 50,000 and are similar in specific activity. Isozymes I to IV contained two polypeptide chains each. The light and heavy chains have $M_r = 15,000$ and 35,000, respectively. Isozyme V is a single polypeptide. The molecular weight of the sixth isozyme is about 100,000 and it has only 5% of the specific activity of the other isozymes. On Ouchterlony immunodiffusion, an anti-serum formed precipitin lines against the urea-denatured isozyme with $M_r = 100,000$. This immunoreactivity showed immunoidentity with those formed against other isozymes.

The N-terminal sequence of light chains was identical for the isozymes. This sequence is homologous to the N-terminal sequence of other acid proteases, especially near the region of the active center aspartate-32. The N-terminal sequence of the single chain, isozyme V, is apparently the same as the light chain sequence. The N-terminal sequence analysis of the heavy chain from isozyme I produced two sets of related sequences, suggesting the presence of structural microheterogeneity.

The carbohydrate analysis of the isozymes, the light chain, and the heavy chain revealed the presence of possibly four attachment sites, with one in the light chain and three in the heavy chain. Each carbohydrate unit contains 2 residues of mannose and 1 residue of glucosamine.

The results suggest that the high molecular weight cathepsin D ($M_r = 100,000$) is the probable precursor of the single chain ($M_r = 50,000$), which in turn produces the two-chain isozymes. These are likely in vivo processes.

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In the past few years, the structure-function relationships of extracellular acid proteases, such as pepsin, have become better established due to the availability of information on the primary and tertiary structures and on the location of the active center (5, 6). This prompted an enhanced interest in comparison of these extracellular acid proteases with cathepsin D at the structure-function level. To accomplish this, relatively large amounts of cathepsin D would be needed. We have, therefore, attempted to develop a large scale purification method for cathepsin D. In a previous paper (7), we reported that the cathepsin D isozymes from porcine spleen are suitable for purification, and that the properties of these isozymes are similar to those of pepsin as well as other extracellular acid proteases. We describe here a large scale purification procedure for all of the cathepsin D isozymes from porcine spleen. With these purified isozymes, the polypeptide arrangements, active site locations, the carbohydrate units, and amino acid sequence homology with pepsin are now known and are described in this report.

MATERIALS

Porcine spleens were obtained from Wilson Packing Co. (Oklahoma City, OK) and kept at $-20^\circ$C. Neuraminidase (Lot 574448), $\alpha$-chymotrypsin (Lot 564402X), and bovine serum hemoglobin were obtained from Worthington. Con A-Sepharose (Lot 5761), DEAE-Sepharose A-25, and Sepharose 4B were purchased from Pharmacia. Wheat germ lectin-Sepharose 6MB (Lot 98C-0433), diisopropylfluorophospho-fluorophosphate, phenylmethylsulfonyl fluoride, 5,5'-dithiobis(2-nitrobenzoic acid), cytochrome c, bovine serum albumin, and diazoacetyl-($\varepsilon$-nor-lysine methyl ester (Lot 116C-0130) were purchased from Sigma. Ovalbumin was obtained from Miles. N-hydroxy-succinimide and 1,6-hexanediamine were purchased from Aldrich, N,N'-dicyclohexyl-carbodiimide was obtained from Pierce. Ampholine, pf 5 to 8, was purchased from LKB. Pepstatin A and leupeptin were obtained from Protein Research Foundation, Osaka, Japan. Sequenator reagents of sequencer grade were purchased from Beckman and amino acid analyzer reagents were purchased from Durrum. All other chemicals were reagent grade. Porcine renin was obtained from Nutritional Biochemicals.

METHODS

Enzyme Assay—Cathepsin D activity was measured with hemoglobin as substrate in a procedure similar to the method of Anson (8). Two milliliters of 0.25 M sodium formate buffer, pH 5.2, containing various amounts of cathepsin D was mixed with 0.5 ml of 5% bovine hemoglobin in distilled water. After incubation at $37^\circ$C for 20 min, 2 ml of 10% trichloroacetic acid was added to precipitate proteins. After filtration through Whatman No. 50 paper, the absorbance at 280 nm was measured. The controls were carried out with incubation mixtures to which cathepsin D was added before the addition of trichloroacetic acid.

$\varepsilon$-Aminoornithine; DFP, diisopropyl phosphorofluoridate; DMAA, Na,N,N'-dinitrobis-l-lysine; Pch$_2$SO$_4$, phenylmethylsulfonyl fluoride; PTH, the phenylthiohydantoin derivative of an amino acid; SDS, sodium dodecyl sulfate; TLCK, N-p-tosyl-L-lysine chloromethyl ketone; TPCK, L-1-tosylamide-2-phenylethyl chloromethyl ketone.
acid. One unit of enzyme activity is defined as the net extinction value of 1.0 of the filtrate in excess of the control. All quantitative determinations were carried out in the linear range of the assay, up to a net absorbance of 0.3.

**Protein Determination**—The protein concentration was determined by absorbance at 280 nm or by the method of Lowry et al. (6) using bovine serum albumin as the standard.

**Incorporation of Diazooacetyl-d-glucopyranosyl Sulfide into the Heavy Chain of Cathepsin D**—The reaction of diazoacetyl-d-glucopyranosyl sulfide with cathepsin D was carried out as described by Rajagopalan et al. (10). The molar ratios of cathepsin D, cupri ions, and the diazo reagent were 1:100:100. Cathepsin D was estimated using the anthrone method (11). Neutral and amino acids were determined by gas-liquid chromatography according to the procedure of Griggs et al. (12) with some modifications (13). The reduced sugars were acetylated in a mixture of 1.5 ml of pyridine and 1.5 ml of acetic anhydride at 80°C for 10 min. The aldoluc acetates were extracted with chloroform and analyzed in a Pakard-Beckec chromatograph equipped with dual flame ionization detectors.

**Automated Edman Degradation**—Analyses were carried out in a Beckman sequencer model 890C using Beckman 102974 fast peptide-DNAma program, as previously described (14). Identifications of PTH-derivative and their trimethylsilyl derivatives were by gas-liquid chromatography in a Beckman GC-65 chromatograph with glass column and SP-400 packing. Thin layer chromatography (15) was also performed for further identification of PTH-derivative.

**Chromatography on Columns of Con A-Sepharose and Wheat Germ Lectin-Sepharose**—A xylene extract (50 ml) of Con A-Sepharose and wheat germ lectin-Sepharose was equilibrated at 4°C with 0.05 M Tris-HCl buffer, pH 7.0, containing 0.2 M NaCl and 1 mM concentration each of CaCl2, MgCl2, and MnCl2. Cathepsin D was eluted at room temperature with 0.2 M a-methyl-d-glucopyranoside in the same buffer. The chromatography on a wheat germ lectin-Sepharose column (1 ml) was carried out under identical conditions.

**Automated Dowmer Degradation**—Double immunodiffusion was carried out in 1% agarose in 0.9% NaCl. The concentrations of antigens were about 0.5 mg/ml in distilled water. The precipitin lines usually appeared after an overnight diffusion at room temperature.

**Amino Acid Analyses**—The amino acid analyses were performed according to the method of Spackman et al. (16) with a Durrum model D500 amino acid analyzer. Protein samples for amino acid analysis were hydrolyzed with 3.7 n HCl for 24, 48, and 72 h at 108°C in evacuated sealed tubes. Half-cystine residues were determined from the cystine peak and also as cysteic acid from a separate analysis after performic acid oxidation and hydrolysis (17). The values for serine and threonine were corrected for decomposition by extrapolation to the body time of hydrolysis. Tryptophan was determined spectrophotometrically (18). The determination of free sulhydryl in cathepsin D was carried out by titration with 5,5'-dithiobis(2-nitrobenzoic acid) (19).

**Preparation of Antiserum against the Heavy Chain of Porcine Cathepsin D**—The rabbit antiserum was prepared by Dr. Ann H. Erickson of Rockefeller University. The antiserum was collected 148 days after two injections with adjuvant of approximately 1.25 mg M. HS04 for 1 h at 80°C, after which the sialic acid content of cathepsin D was estimated using the anthrone method (11). Neutral and amino acid contents were extracted with chloroform and analyzed in a Packard-Becker model 420 gas chromatograph equipped with dual flame ionization detectors.

**Preparation of Isozyme V from Polyacrylamide Gels**—Crude isozyme V (1 mg) from isoelectric focusing was subjected to SDS-Polyacrylamide gel electrophoresis in a Tris-glycine buffer system (pH 8.3), as was performed according to the method of Davis (22). SDS-polyacrylamide gel electrophoresis was carried out according to Weber and Osborn (23). Before SDS gels at 4°C, the protein samples were dissolved in SDS (0.1%) buffer containing 0.1% β-mercaptoethanol and heated at 100°C for 5 min. Gels were stained with 0.2% Coomassie R 250 in 50% methanol at 65°C for 1 h and destained in 7% acetic acid at 65°C overnight.

**Preparation of Isozyme V from Polyacrylamide Gels**—Crude isozyme V (1 mg) from isoelectric focusing was subjected to SDS-Polyacrylamide gel electrophoresis. Twenty gels were used. After electrophoresis, one gel was stained. The regions corresponding to Mr. 50,000 were then cut out from the rest of the gels. The finely cut gel was placed inside a glass tube, to which a 2-cm closed dialysis tubing was firmly attached. The tube was filled with the electrophoresis buffer and placed in the electrophoresis bath with the dialysis tubing at the anode side. The electrophoresis was performed at 8 mA/gel for 16 h. The solution in the dialysis tubing was thoroughly dialyzed against distilled water and lyophilized. The isozyme V thus obtained was used for NH2-terminal sequence analysis and amino acid analysis.

**RESULTS**

**Large Scale Purification of Cathepsin D**—The scheme for large scale purification of cathepsin D from porcine spleen is shown in Table I. The experimental conditions are described in detail in a miniprint supplement accompanying this manuscript. The first three steps were similar to the procedures which we reported previously (7). Since large amounts of proteins were obtained (290 g from 2 kg of tissue), precise conditions for Steps 4, 5, and 6 (see miniprint supplement) were critical in both the yield and the purity. The elution patterns from DEAE-Sephadex and peptatin affinity columns are also shown in the miniprint supplement (Fig. 1B). Up to this point, about 11% yield with 410-fold purification was obtained.

We have previously reported that at least three isozymes of cathepsin D are present in the porcine spleen (7, 24). The isolation of five isozyme forms was achieved in Step 7 with preparative isoelectric focusing (Fig. 1). These cathepsin D was estimated using the thiorbitaric acid method as described by Warren (20).

**Analytical Isoelectric Focusing**—Isoelectric focusing in polyacrylamide gels was carried out with a Protean apparatus (21). Protein samples (50 µg to 100 µg) were mixed with gel solution. The focusing was started at 1 mA/gel. In approximately 60 min the voltage was gradually increased to 350 V while 1 mA/gel was maintained. After focusing for 2½ h at 350 V, the gels were stained with 0.04% Coomassie G in 3.5% perchloric acid overnight and destained in 3.5% perchloric acid for 30 min. The gels were then kept in a solution of 7% acetic acid.

**Polvacrylamide Gel Electrophoresis**—Polvacrylamide gel electrophoresis in a Tris-glycine buffer system, pH 8.3, was performed according to the method of Davis (22). SDS-polyacrylamide gel electrophoresis was carried out according to Weber and Osborn (23). Before SDS gels at 4°C, the protein samples were dissolved in SDS (0.1%) buffer containing 0.1% β-mercaptoethanol and heated at 100°C for 5 min. Gels were stained with 0.2% Coomassie R 250 in 50% methanol at 65°C for 1 h and destained in 7% acetic acid at 65°C overnight.

**Results**

**Table I**

<table>
<thead>
<tr>
<th>Step No.</th>
<th>Total enzyme activity mg</th>
<th>Total protein mg</th>
<th>Specific activity</th>
<th>Yield</th>
<th>Purification fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Homogenate</td>
<td>290,773</td>
<td>21,808</td>
<td>0.075</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>2. Supernatant</td>
<td>89,684</td>
<td>11,076</td>
<td>0.124</td>
<td>50.79</td>
<td>1.63</td>
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<tr>
<td>3. Acid supernatant</td>
<td>43,412</td>
<td>8,335</td>
<td>0.192</td>
<td>38.22</td>
<td>2.56</td>
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<tr>
<td>4. Lyophilized powder</td>
<td>20,282</td>
<td>5,653</td>
<td>0.279</td>
<td>25.92</td>
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<td>5. DEAE-Sephadex</td>
<td>14,993</td>
<td>5,088</td>
<td>0.361</td>
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<td>4.81</td>
</tr>
<tr>
<td>6. Pepstatin-Sephadex</td>
<td>77</td>
<td>2,389</td>
<td>0.307</td>
<td>10.86</td>
<td>410.27</td>
</tr>
<tr>
<td>7. Isoelectric focusing</td>
<td></td>
<td></td>
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<td></td>
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</tbody>
</table>
isozymes will be referred to as isozymes I to V in the order of their pi values, which are 7.5, 7.1, 6.6, 6.1, and 5.5, respectively.

One special note should be made on the apparent low recovery, about 50%, from the pepstatin-Sepharose column in Step 6. As reported in a preliminary communication (24), this apparent loss of total activity was due to the separation of "activator" proteins from cathepsin D at this step. The breakthrough material contained no cathepsin D activity (miniprint supplement, Fig. 2S). However, on reconstitution of this fraction with purified cathepsin D (second peak in Fig. 2S), the apparent proteolytic activity was enhanced about 2-fold (results not shown). The detailed results on the "activator" proteins are not within the scope of the current report. For the present discussion, it is important to point out that the recovery in Step 6 is nearly quantitative, and that taking into account the effect of the "activator" proteins, the overall purification in Table I should be nearly 800-fold. The isozyme forms and their properties, to be described in the following sections, are summarized in Table II.

Separation of Isozyme V and a High Molecular Weight Form of Cathepsin D—The isozymes I, II, III, and IV obtained from isoelectric focusing (Fig. 1) were essentially pure (see section on homogeneity). The material obtained under Peak V (Fig. 1), however, contained several components, as judged from the patterns of SDS polyacrylamide gel electrophoresis, having molecular weights of about 100,000, 50,000, 35,000 (minor yield), and 15,000 (minor yield) (results not shown). This material was then further fractionated over a column of Sephadex G-100. The protein peak eluted at the void volume (Fig. 2) contained only the cathepsin D of $M_r = 100,000$. (This species will be referred to as the high molecular weight cathepsin D.) A second peak, which was eluted in the area corresponding to a molecular weight of 50,000, gave rise to three bands in the SDS gel electrophoresis. The bands corresponding to molecular weights 35,000 and 15,000 are the heavy and light chains of cathepsin D, probably from the minor contamination of isozyme IV (see "Subunits"). The band which gave $M_r = 50,000$ was apparently the single chain isozyme. Since this isozyme (V) is present in low quantity, further purification was carried out only following elution of this band from several gels after electrophoresis (see "Methods"). Isozyme V isolated in this manner had no enzymic activity, but was useful in further chemical characterization.

Proteolytic Activity of the Isozymes—The specific proteolytic activity of isozymes I, II, III, and IV are the same with hemoglobin as substrate. Isozyme V, recovered from Sephadex G-100 with some contamination of isozyme IV, also had the same specific activity. The high molecular weight cathepsin D, however, has a relatively low specific proteolytic activity.

<table>
<thead>
<tr>
<th>Table II</th>
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<tbody>
<tr>
<td>Summary on the properties of cathepsin D isozymes from porcine spleen and comparison with the isozymes from bovine spleen*</td>
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<tr>
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<td>pI</td>
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<td></td>
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<td></td>
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<tr>
<td>Porcine isozyme</td>
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<td>II</td>
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<td>III</td>
</tr>
<tr>
<td>IV</td>
</tr>
<tr>
<td>V</td>
</tr>
<tr>
<td>Light chain</td>
</tr>
<tr>
<td>Heavy chain</td>
</tr>
<tr>
<td>Bovine isozyme*</td>
</tr>
<tr>
<td>A</td>
</tr>
<tr>
<td>B</td>
</tr>
<tr>
<td>Light chain</td>
</tr>
<tr>
<td>Heavy chain</td>
</tr>
</tbody>
</table>

* Data taken from a separate study (31).

+ Data are from amino acid analyses after reacting the native enzyme with diazoacetyl norleucine methyl ester.

+ Man and GlcN are mannose and glucosamine, respectively.

+ Only trace reactivity observed.

(Extract from the original document)
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of about 5% of the other isozymes. This point is clearly illustrated in the relative specific activities under the two peaks in Fig. 2.

Homogeneity and Molecular Weights—Three major isozyme (I, II, and III) preparations contained only a single isoelectric species each (Fig. 3S). All isozymes showed a single peak on Sephadex G-100 column chromatography, \( M_r = 50,000 \) (Fig. 4S). Because different molecular weights have been reported for cathepsin D (4), careful determinations were conducted. A value of \( M_r = 50,000 \) was obtained when the chromatographic experiments were carried out either separately or together with the marker proteins, using a long column. Additionally, a number of other homogeneity criteria were obtained. On SDS gel electrophoresis, isozymes I, II, III, and IV each produced two bands which will be shown in the following section to be subunits. The combined molecular weights of the two chains are also 50,000, in agreement with the Sephadex column chromatography. These results also indicate the absence of non-cathepsin D contaminants in these four isozyme preparations. The amino acid compositions of the isozymes (see below) are almost identical and further indicate the absence of impurity. Finally, the NH\(_2\)-terminal sequences of the isozymes could be accounted for from that of the isolated subunits, which also indicates homogeneity. Isozyme V recovered by elution of SDS-polyacrylamide gels gave a single band when again subjected to electrophoresis (Fig. 3a).

The high molecular weight cathepsin D isozyme eluted from Sephadex G-100 (Fig. 2) gave a single band on SDS gel electrophoresis (Fig. 3b) with \( M_r = 100,000 \). On disc gel electrophoresis, this isozyme produced one major sharp band and a slightly faster moving minor band (Fig. 3b). Whether the presence of two bands represents the presence of impurity is not yet clear. This point must be further clarified when this isozyme species can be purified in larger quantity.

Subunits—Porcine cathepsin D isozymes I, II, III, and IV are each composed of a heavy chain \( (M_r = 35,000) \) and a light chain \( (M_r = 15,000) \) (Fig. 4). SDS gel electrophoresis of isozymes I to IV in the presence of a reducing agent produced the same pattern. This indicates that the subunits are not linked through a disulfide bond.

The separation of the chains from isozyme I was achieved with a column of Sephadex G-75, using a buffer containing 6 M urea or 5 M guanidine HCl (Fig. 5a). From the electrophoretic mobility, the peaks in Fig. 5a were identified to be the heavy chain and the light chain, respectively (Fig. 5b). The chains from the other two-chain isozymes (II, III, and IV) can also be isolated using the same conditions (results not shown).

The isolated light and heavy chains each produced a single band in standard polyacrylamide electrophoresis (results not shown). The electrophoretic mobility of the light chains ob-
Cathepsin D Isozymes from Porcine Spleens

The heavy chains had different mobilities. This is consistent with the results illustrated in Fig. 5A. The mixture of heavy chains from all isozymes showed multiple electrophoretic bands as for native cathepsin D. The light chain obtained from the isozyme mixture produced a single band.

Amino Acid Composition—The amino acid compositions of cathepsin D isozymes from porcine spleens are given in Table III. The detailed statistics of the data are given in Table VII.

Carbohydrate Contents—Several pieces of evidence indicated that the cathepsin D isozymes are glycoproteins. After

**Table III**

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Porcine Isozymes</th>
<th>Porcine Isozyme</th>
<th>Bovine Isozymes</th>
<th>Bovine chymosin</th>
<th>Bovine H-chain</th>
<th>Porcine H-chain</th>
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<td></td>
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<td>II</td>
<td>III</td>
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**Fig. 5. Isolation of cathepsin D heavy and light chains.** a, Separation of heavy chain and light chains of porcine cathepsin D on a column of Sephadex G-75 (1.5 × 90 cm). The elution was carried out with 0.05 M sodium acetate, pH 5.2, containing 6 M urea. b, SDS gel electrophoretic pattern of heavy chain (first peak) and light chain (second peak) from porcine cathepsin D.
electrophoresis the cathepsin D band gave a positive stain with periodate-Schiff reagent (25) on polyacrylamide gel (results not shown). Also, this enzyme was retained by a column of Con A-Sepharose and recovered by elution with a buffer containing 0.2 M α-methylglucopyranoside (Fig. 6A). Cathepsin D formed a precipitate line with Con A in agarose diffusion (Fig. 6B, inset). The overall carbohydrate content of cathepsin D was estimated to be about 5%. For cathepsin D I, II, and III, each isozyme molecule contains 8 residues of mannose and 4 residues of N-acetylglucosamine (Table IV). No significant amounts of sialic acid or other sugars were found.

Because of insufficient material, isozymes IV and V were not analyzed for carbohydrates. Cathepsin D was found not to be bound by wheat germ lectin-Sepharose (results not shown), suggesting that mannose residues occupy the nonreducing terminal of the carbohydrate units in the enzyme molecules. About 75% of the carbohydrate of the native cathepsin D was found in the heavy chain (Table IV). The mannose to glucosamine ratios (about 2:1) are the same in both the light and heavy chains. The carbohydrate in the light chain contains only 2 residues of mannose and 1 residue of N-acetylglucosamine. From the carbohydrate structure of some other glycoproteins (26, 27), the general order is from mannose to N-acetylglucosamine and then to the side chain of asparagine. Based on these common structural patterns, it might be tentatively proposed that the structure of this single carbohydrate unit in the light chain is mannose-mannose-GlcNAsn. If these same structural units are present in the heavy chain, then three carbohydrate attachment sites should be present.

**Immunological Relationships of Cathepsin D Isozymes**—An antiserum raised from the cathepsin D heavy chains was tested against various isozymes from porcine spleens. Porcine cathepsin D isozymes I, II, III, and IV showed complete immunological identity in Ouchterlony double diffusion (Fig. 6A). The antiserum formed only very weak precipitin lines with the isolated light chains, but not with renin, another acid protease produced in kidney. As expected, the antiserum forms precipitin lines with the heavy chains.

Most interestingly, the antiserum formed precipitin lines against high molecular weight cathepsin D ($M_\text{r} = 100,000$) after it had been denatured in 8 M urea. As shown in Fig. 6B, the immunological reactivity of this species was identical with that produced by other cathepsin D isozymes. The native high molecular weight isozyme, when diffused in the absence of urea, had no immunoactivity with the antiserum. This essentially excluded the possibility that the reactivity was due to contamination by small amounts of other isozymes.

**Reactive Site of Diazoo Inactivator**—Pepsin and other acid proteases contain two aspartic acids which can be specifically reacted with active site-directed reagents (5). An epoxide inactivator specifically modifies aspartate-32 (28, 29), and a number of diazo inactivators specifically esterify aspartate-215 (29, 30). Cathepsin D is known to be inactivated by both types of reagents.

With either an isozyme mixture or isozyme I, diazoacetyl norleucine methyl ester incorporated 1 residue of norleucine/molecule of cathepsin D ($M_\text{r} = 50,000$). With the diazo-inactivated isozyme I, 1 residue of norleucine was found on the amino acid analysis of the heavy chain, and no norleucine was found in the light chain (Table II). As will be described below, an aspartyl residue had been found at position 32 of cathepsin D with a surrounding sequence very similar to the active site sequence near the aspartate-32 of other acid proteases. Since this is the most probable site of epoxide esterification, the reaction of cathepsin D with this inactivator was not further studied.

**NH$_2$-terminal Sequences of Cathepsin D Isozymes**—Table V shows that a sequence of 38 residues was obtained for porcine isozyme I light chain. The sequence identifications and quantitative data are in the miniprint supplement. Light chains from other cathepsin D isozymes (II, III, and IV) have sequences identical with that from isozyme I. The NH$_2$-terminal sequence from the light chain of bovine spleen cathepsin D, taken from separate work (31), is also shown in Table V for comparison. The only substitution in the first 34 residues was found at position 5 (pepsin residue numbers). It is apparent from the alignment in Table V that these two cathepsin D sequences are highly homologous with the NH$_2$-terminal sequences of other acid proteases. Especially striking are the sequences near the active center aspartate-32.

The amino acid sequences in the NH$_2$-terminal region of heavy chain preparations always contained two major sets of sequences. (Sometimes a minor third sequence could also be

### Table IV

<table>
<thead>
<tr>
<th>Carbohydrate composition of porcine cathepsin D isozymes and subunits</th>
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<tbody>
<tr>
<td>Carbohydrate</td>
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<tr>
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<tr>
<td>Mannose</td>
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<td>Glucosamine</td>
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Fig. 6. Ouchterlony immunodiffusion patterns of porcine cathepsin D isozymes on agarose plates. **Plate A**, the center well contained antiserum against porcine cathepsin D heavy chains. Purified isozymes I, II, III, and IV were placed in wells a, b, c, and d, respectively. **Plate B**, the center well contained the same antiserum. Well 1 contained the high molecular weight isozyme which had been treated with 8 M urea. Well 2 contained a mixture of cathepsin D isozymes.
**Cathepsin D Isozymes from Porcine Spleens**

**TABLE V**

NH2-terminal sequence of cathepsin D light chains and their homology to gastric and microbial acid proteases

<table>
<thead>
<tr>
<th>Residue numbers</th>
<th>2</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
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<th>17</th>
<th>18</th>
<th>19</th>
<th>20</th>
</tr>
</thead>
<tbody>
<tr>
<td>Porcine cathepsin D</td>
<td>Gly-Pro-Ile-Pro-Glu-Val-Leu-Lys-Asn-Tyr-Met  &amp; Asp-Ala-Gln-Tyr-Tyr-Gly-Ile-Gly-Ile-</td>
<td></td>
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<tr>
<td>Bovine cathepsin D</td>
<td>Gly-Pro-Ile-Pro-Glu-Leu-Leu-Lys-Asn-Tyr-Met  &amp; Asp-Ala-Gln-Tyr-Tyr-Gly-Ile-Gly-Ile-</td>
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<td><strong>Gastric Proteases:</strong></td>
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<tr>
<td>Porcine pepsin (5)</td>
<td>Ala-Leu/Ile-Gly-Asp-Glu-Pro-Leu-Glu-Asn-Tyr-Leu  &amp; Asp-Thr-Glu-Tyr-Phe-Gly-Thr-Ile-Gly-Ile-</td>
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<tr>
<td>Bovine chymosin (44)</td>
<td>Gly-Glu-Val-Ala-Ser-Val-Thr-Asp-Asn-Asp-Asp-Glu-Val-Ile-Thr-Pro-Val-Thr-Ile-</td>
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<tr>
<td><strong>Microbial Proteases:</strong></td>
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<tr>
<td>R. chinensis (14)</td>
<td>Ala-Leu/Ile-Gly-Asp-Glu-Pro-Leu-Glu-Asn-Tyr-Leu  &amp; Asp-Thr-Glu-Tyr-Phe-Gly-Thr-Ile-Gly-Ile-</td>
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<tr>
<td>Penicillopepsin (6)</td>
<td>Gly-Glu-Val-Ala-Thr-Asn-Thr-Pro-Asp-Tyr-Gly-Ile-Thr-Pro-Val-Ile-</td>
<td></td>
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</tr>
</tbody>
</table>

| Residue numbers | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 | 31 | 32 | 33 | 34 | 35 | 36 | 37 |
|-----------------|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| Porcine cathepsin D | Gly-Thr-Pro-Pro-Gln-Ser-Phe-Thr-Val-Phe-Asp-Thr-Gly-Ser-Ser-Asn- |
| Bovine cathepsin D | Gly-Thr-Pro-Pro-Gln-Ser-Phe-Thr-Val-Phe-Asp-Thr-Gly-Ser-Ser-Asn- |
| **Gastric Proteases:** | | | | | | | | | | | | | | | | | | | | | |
| Porcine pepsin | Gly-Thr-Pro-Pro-Gln-Asp-Thr-Val-Ile-Asp-Asp-Glu-Thr-Ile-Thr-Pro-Val-Thr-Ile- |
| Bovine chymosin | Gly-Thr-Pro-Pro-Gln-Asp-Thr-Val-Ile-Asp-Asp-Glu-Thr-Ile-Thr-Pro-Val-Thr-Ile- |
| **Microbial Proteases:** | | | | | | | | | | | | | | | | | | | | | |
| R. chinensis | Gly-Thr-Pro-Pro-Gln-Glu-Thr-Val-Phe-Thr-Val-Phe-Asp-Thr-Gly-Ser-Ser-Asp- |
| Penicillopepsin | Gly-Thr-Pro-Pro-Gln-Glu-Thr-Val-Phe-Thr-Val-Phe-Asp-Thr-Gly-Ser-Ser-Asp- |

*The residue numbers used in this table are the pepsin numbers (5). In porcine pepsin, the residues with minus numbers are derived from the sequence of pepsinogen (5).*

*The data on the identification and yield of individual residuals are listed in the miniprint supplement of this manuscript.*

*The bovine cathepsin D sequence is taken from the result of a separate work.*

**TABLE VI**

NH2-terminal sequences of porcine cathepsin D heavy chain

Two sets of sequences were observed in automated Edman degradation. Similarity in the sequences (identical residues in boxes) are seen when one set of the sequence (top) is aligned against the other with a shift of 2 residues.

<table>
<thead>
<tr>
<th>1</th>
<th>Val-Ile</th>
<th>5</th>
<th>Gly-Thr-Pro</th>
<th>10</th>
<th>Gly-Thr-Pro</th>
<th>15</th>
<th>Gly-Pro-Glu-Leu</th>
<th>20</th>
<th>Gly-Leu</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>Gly-Gly</td>
<td>5</td>
<td>Gly-Thr-Pro</td>
<td>10</td>
<td>Gly-Thr-Pro</td>
<td>15</td>
<td>Gly-Pro-Glu-Leu</td>
<td>20</td>
<td>Gly-Leu</td>
</tr>
</tbody>
</table>

observed.) Two sets of observed sequences obtained from the heavy chain of isozyme I (Table VI) appear to be related in sequence homology. In the alignment, the second sequence (about 35%) is 2 residues longer at the NH2 terminus. Other heavy chains appeared to have the same sequences when the first few residues were identified.

The single chain cathepsin D (isozyme V) obtained from a preparative SDS-polyacrylamide gel showed a sequence of Gly-Pro-Ile-. This sequence is identical with the first 3 residues of the light chain, suggesting that the single chain cathepsin D must contain the light chain at the NH2-terminal region.

**DISCUSSION**

The large scale purification of cathepsin D isozymes reported here is a reliable and uncomplicated procedure. We have successfully used this procedure in the past 2 years. From porcine spleen five isozymes with M, = 50,000 and a high molecular weight isozyme with M, = 100,000 were obtained (see Table II). This procedure can also be adopted for the purification of two cathepsin D isozymes from bovine spleen. The results of studies on the bovine enzyme will be reported separately (31).

Porcine spleen isozymes I, II, III, and IV each contain two polypeptide chains, while isozyme V and the high molecular weight isozyme are apparently single chain enzymes. The comparative polypeptide chain arrangements of the isozymes are shown in Fig. 7. The two-chain isozymes are apparently generated from the single chain cathepsin D by peptide bond cleavages. This relationship is similar to that suggested for cathepsin D from human liver (32) and bovine uterus (33). The polypeptide chain arrangements are now clarified. The order of the two chains in the original single chain cathepsin D must have been from light to heavy chain, starting from the NH2 terminus. This is supported by the fact that: (i) the NH2-terminal sequences of the light and single chains are
Cathepsin D Isozymes from Porcine Spleens

The NH\textsubscript{2}-terminal structure of light chains is highly homologous with the NH\textsubscript{2}-terminal portion of pepsin (Table V); and (iii) the active center aspartate-32 is found in the light chain (Table V), while the diazo-reactive site is in the heavy chain (presumably Asp-215).

The porcine spleen cathepsin D isozymes, however, are clearly different from the cathepsin D of rat and mouse spleen (34) which contains monomeric and disulfide-linked dimeric forms. The porcine spleen cathepsin D isozymes (including the high molecular weight isozyme) do not produce lower molecular weight forms upon treatment with sulfhydryl reagents.

The two NH\textsubscript{2}-terminal sequences of heavy chain suggested that they may be the result of different gene products. As shown in the alignment in Table VI, two sets of sequences obtained from porcine isozyme I heavy chain differ by a length of 2 residues at the NH\textsubscript{2} termini. Even though the similarity of the two sets of sequences is very clear, the differences (7 out of 19 residues) can be explained by their probably being the products of different genes. It is interesting that in contrast to the microheterogeneity of the heavy chains, only a single sequence was found in the NH\textsubscript{2}-terminal region of light chains. This may be due to a more important structural or functional role of this region, which is thus more conserved in the evolution of this protein. We have also studied two isozymes from bovine spleen (31). The chain arrangements in the bovine cathepsin D isozymes are basically similar to those of porcine origin, even though the molecular weights of the individual chains are slightly different from their porcine counterparts (Table II). Also, bovine cathepsin D contains about 65% of the single chain species as contrasted to less than 5% in porcine cathepsin D. In both animal species, however, the number of spleens required for the purification was large, and, inevitably, they must come from animal populations of heterogeneous genetic origin. At present we do not know whether the structurally distinguishable isozyme forms are found in a single animal.

The main structural reasons for the different isoelectric points of the isozymes are not clear. It has been suggested to be due to differences in sialic acid contents (35). However, the carbohydrate analysis (Table IV) showed clearly the absence of sialic acid. This evidence was supported by the observation that the electrophoretic mobilities of the isozymes were unchanged after the treatment of neuraminidase (results not shown). The difference in pI values can apparently be traced to the heavy chain region on the cathepsin D molecule. The light chains isolated from different isozymes were indistinguishable in analytical isoelectric focusing. However, the microheterogeneity at the NH\textsubscript{2}-terminal region of the heavy chain does not appear to be the decisive factor which determines the pI values of the isozymes. This is clearly illustrated by the fact that each of the two-chain isozymes with different pI values contains the same two sets of amino acid sequences at the NH\textsubscript{2} termini of the heavy chains. Finally, incubation of individual cathepsin D isozymes at pH 3.2 and 4.2 did not produce another isozyme form with a different pI value (results not shown), suggesting that the different pI forms are not generated by autodigestion of the enzyme. At this time we can only suggest that there are yet unobserved structural differences in these isozymes which caused the differences in pI values.

We have also considered another important question: whether the two-chain isozymes are produced by proteolysis during the homogenization of tissue or subsequent purification steps. The following observations suggest that this is not the case. (a) When several protease inhibitors such as DFP (1 mM), PhCH\textsubscript{2}SO\textsubscript{2}F (1 mM), TLCK (0.5 mM), TPCK (1 mM), soybean trypsin inhibitor (1 mg/ml), leupeptin (0.1 mM), and EDTA (10 mM) were included in the homogenization and in the purification procedures of cathepsin D, the electrophoretic pattern of cathepsin D was identical with that of cathepsin D purified in the absence of these inhibitors (Fig. 1). (b) Replacement of acid precipitation (Step 3 in Table I) with 35 to 70% ammonium sulfate fractionation also gave the same isozyme pattern on isoelectric focusing. This indicated that the acid precipitation in Step 3 did not produce the two-chain species.
that shown in Fig. 1 was again observed. (d) When cathepsin D was first inactivated with pepstatin and then subjected to isoelectric focusing, an identical isozyme profile as that shown in Fig. 1 was obtained. In all of these experiments, if the content of the single chain isozyme was increased by various treatments, an elevated peak corresponding to isozyme V (single chain) would have been seen with a concomitant decrease of other isozymes. From this evidence, we tentatively suggest that both the single and the two-chain species of cathepsin D are present in vivo.

We have shown previously that the specific pepsin inactivator, 1,2-epoxy-3-(p-nitrophenoxyl)propanol, also inhibits cathepsin D (7). In pepsin the site of esterification is on the side chain of an aspartate residue at position 32 (28). The sequence around this residue in cathepsin D shows homology to that of pepsin (Table V). It seems most probable that aspartate-32 in cathepsin D is also the site of esterification by the epoxide inactivator. Diazooacetyl norleucine methyl ester, another pepsin inactivator (4), apparently reacts with cathepsin D at a single site, as the incorporation of norleucine was 1 mol/mol of cathepsin D. The fact that the incorporation occurred in the heavy chain is consistent with the known pepsin reactive site at aspartate-215 (5). These results are also consistent with the findings of Keilova (36), who reported that the peptide which contained the diazo-reactive aspartate residue had an identical sequence with that around aspartate-215 of pepsin.

The carbohydrate analysis showed that cathepsin D contains 8 mannose and 4 glucosamine residues/molecule. There are possibly four sites per molecule for carbohydrate attachment. One of the sites is located on the light chain, while the others are on the heavy chain. Each carbohydrate unit may be attached through an asparagine side chain in a structure mannos-mannose-N-acetylglucosamine-Asn. This suggested structure is consistent with the binding of cathepsin D to Con A-Sepharose gels and with the lack of interaction with wheat germ lectin. The precise structure and the glycosidic linkages remain to be determined. If the common structural pattern of carbohydrates in glycoproteins (26, 27) prevails in cathepsin D, the linkages of the nonreducing terminal mannose to the internal mannose is possibly α(1-3), while the linkage between mannose and N-acetylglucosamine may be β(1-4). In contrast to previous suggestions (35), no sialic acid was found in cathepsin D. It is interesting to note that the absence of sialic acid in several other lysosomal enzymes has been reported (37-39). To the best of our knowledge a carbohydrate unit as simple as this one has not been proposed for another glycoprotein. Additionally, an identical 2:1 ratio for mannose and N-acetylglucosamine has been reported for other lysosomal enzymes (39-42). It seems probable that other lysosomal enzymes may contain the same carbohydrate structural units as in cathepsin D.

With the similarity of sequences and active sites of cathepsin D and other acid proteases, it can be predicted that this lysosomal enzyme has a tertiary structure and catalytic mechanism very analogous with other acid proteases. A common evolutionary origin of cathepsin D with the gastric and other acid proteases is probable. However, cathepsin D is unique among the acid proteases in that its molecular weight is near 50,000, as contrasted to about 35,000 for all of the others. It had been previously reported that cathepsin D has a molecular weight of 35,000 (7). However, the low figure was actually obtained from the heavy chain on an SDS gel. The light chain stained much weaker when low amounts of protein were used and was mistakenly thought to be a minor impurity band. Also, Sephadex G 200 was previously used for molecular weight determination. The estimate was considerably less accurate when compared to the result with Sephadex G-100 in this study. We believe that a molecular weight of 50,000 for the porcine cathepsin D is correct. As compared in Table II, the bovine isozymes were found to have M, = 46,000 (31), closer to the value of 42,000 suggested by Barrett (4).

As schematically shown in Fig. 7, porcine cathepsin D as a single polypeptide chain of 50,000 is about 140 residues longer than pepsin (shaded area in Fig. 7). The difference seems too big to be simply a result of residue insertions or deletions in the homologous regions during evolution. It is most likely that cathepsin D contains an extra length (tail) of more than 100 residues at the COOH-terminal end, extending beyond the aligned position for the pepsin COOH terminus. Since the specificity of cathepsin D is very similar to that of other acid proteases, the tail region could have other functions in the regulatory purposes. We have made an estimation of the approximate amino acid composition in the tail region based on the homology to gastric proteases. The composition of light chains from cathepsin D is very similar to that of residues 1 to 100 of bovine chymosin (Table II). If this similarity prevails throughout the length of the chymosin molecule (see Fig. 7), then the composition of the tail region can be reasonably represented by the difference in the composition of the heavy chain and chymosin residues 101 to 328. Such calculations are shown in the last two columns of Table III. Interestingly, large numbers of basic amino acids and hydrophobic residues are found in the difference which represents the tail region. In the hydrophobic group, the contents of proline, valine, isoleucine, and leucine are particularly prominent. We tentatively postulate that the tail region may have membrane binding affinity.

As shown under “Results,” all cathepsin D isozymes appeared identical in immunoreactivity against antiserum to the heavy chains. It is possible that the structural microheterogeneity in the observed genetic variants, as seen in the two NH2-terminal sequences of the heavy chains, is limited to a relatively small region of the molecule; the structural determinants for the immunorecognition may depend entirely on part of the sequence which is common for both demonstrated genetic variants. The fact that it is difficult to separate cathepsin D with different structures representing microheterogeneous variants, either as the native isozymes or as the heavy chains, also supports the view that the structural differences are small in the two observed variants.

One of the most interesting isozymes obtained from porcine spleen in this study is the high molecular weight cathepsin D (M, = 100,000). This isozyme has about 5% of the specific activity of the other isozymes and a complete immunochromatid identity against the heavy chain antisem when denatured (Fig. 6B). Since this species is present in very low amounts in the tissue extract, it has not yet been possible to carry out a comparative structural study. But it seems reasonable to suggest that the high molecular weight isozyme is the precursor form of the single chain species (isozyme V), which is in turn being converted to the two-chain enzyme. The relationships of these three isozyme species are summarized in Fig. 7. We tentatively suggest that these conversions are in vivo processes. It is interesting to note that another enzyme with acid protease characteristics, renin, also has large molecular weight precursors (43). It seems possible that there may be common pathways in the synthesis and activation of these two enzymes.

Acknowledgments—We would like to thank Mrs. Azar Fosniro for skillful technical assistance, Mr. Hamid Naraghi and Mr. Abdul
REFERENCES

38. Additional references are found on p. 11415.
Cathepsin D Isozymes from Porcine Spleens

By S. Huang, S. S. Huang, and D. Tang

MATERIALS USED IN PAPYRUS-TOOL FORMATION OF CATHEPSIN D ISOZYMES

Preparation of Antileukocyte-Spleen (ALS) Cells. Antileukocyte spleen was extracted with 60% w/v glycogen in 100 mM sodium acetate (pH 5.5) and washed twice with 100 mM sodium acetate (pH 5.5). The supernatant was removed, and the precipitate was centrifuged at 10,000 rpm for 30 min. The supernatant was removed, and the precipitate was washed with 100 mM sodium acetate (pH 5.5) three times. The supernatant was removed, and the precipitate was washed with 100 mM sodium acetate (pH 5.5) again. The precipitate was then suspended in 100 mM sodium acetate (pH 5.5) containing 1 M NaCl, 1 M sucrose, and 1 M KCl (starting buffer for affinity chromatography).

Figure 15. Chromatographic pattern of cathepsin D precipitation from preparations of cathepsin D isoforms. The gel was stained with Coomassie Blue G-250 and visualized using a UV transilluminator at 302 nm. The fractions were collected and analyzed for cathepsin D activity by the method described in Experimental Procedures. The fractions were then pooled and concentrated by ultrafiltration.

Figure 21. Chromatographic pattern of cathepsin D isoforms. The gel was stained with Coomassie Blue G-250 and visualized using a UV transilluminator at 302 nm. The fractions were collected and analyzed for cathepsin D activity by the method described in Experimental Procedures. The fractions were then pooled and concentrated by ultrafiltration.
### Amino Acid Composition of Porcine Cathepsin D Isozymes I, II, III, and IV and the Light and Heavy Chains of Isozyme I.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Isozymes residues/mol</th>
<th>Isozymes residues/mol</th>
<th>Isozymes residues/mol</th>
<th>Isozymes residues/mol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
<td>II</td>
<td>III</td>
<td>IV</td>
</tr>
<tr>
<td>Lysine</td>
<td>27.85 ± 0.59 (28)</td>
<td>29.15 ± 0.43 (29)</td>
<td>27.83 ± 0.21 (28)</td>
<td>27.38 ± 0.20 (27)</td>
</tr>
<tr>
<td>Histidine</td>
<td>10.21 ± 0.27 (10)</td>
<td>9.35 ± 0.39 (9)</td>
<td>10.33 ± 0.23 (10)</td>
<td>11.46 ± 0.45 (11)</td>
</tr>
<tr>
<td>Arginine</td>
<td>11.15 ± 0.26 (11)</td>
<td>12.65 ± 0.25 (12)</td>
<td>10.87 ± 0.27 (11)</td>
<td>10.55 ± 0.10 (11)</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>43.14 ± 0.14 (43)</td>
<td>41.33 ± 0.29 (43)</td>
<td>43.33 ± 0.06 (43)</td>
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<tr>
<td>Threonine</td>
<td>27.00 ± 0.18 (27)</td>
<td>27.95 ± 0.14 (27)</td>
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</tr>
<tr>
<td>Serine</td>
<td>30.51 ± 0.58 (31)</td>
<td>30.99 ± 0.48 (31)</td>
<td>31.41 ± 0.12 (31)</td>
<td>30.42 ± 0.59 (30)</td>
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<tr>
<td>Glutamic acid</td>
<td>38.79 ± 0.13 (39)</td>
<td>40.00 ± 0.55 (40)</td>
<td>42.10 ± 0.15 (42)</td>
<td>41.66 ± 0.59 (44)</td>
</tr>
<tr>
<td>Proline</td>
<td>29.27 ± 0.38 (29)</td>
<td>29.69 ± 0.20 (30)</td>
<td>29.06 ± 0.12 (29)</td>
<td>29.63 ± 0.28 (30)</td>
</tr>
<tr>
<td>Glycine</td>
<td>55.37 ± 0.83 (55)</td>
<td>55.55 ± 0.29 (56)</td>
<td>52.82 ± 0.16 (53)</td>
<td>51.87 ± 0.21 (52)</td>
</tr>
<tr>
<td>Alanine</td>
<td>25.11 ± 0.10 (25)</td>
<td>25.04 ± 0.10 (25)</td>
<td>25.00 ± 0.10 (25)</td>
<td>25.61 ± 0.17 (26)</td>
</tr>
<tr>
<td>Half cystine</td>
<td>8.35 ± 0.33 (8)</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Valine</td>
<td>40.17 ± 0.51 (40)</td>
<td>30.36 ± 0.34 (39)</td>
<td>37.61 ± 0.14 (39)</td>
<td>37.56 ± 0.14 (39)</td>
</tr>
<tr>
<td>Methionine</td>
<td>10.90 ± 0.05 (11)</td>
<td>10.99 ± 0.02 (11)</td>
<td>10.28 ± 0.45 (10)</td>
<td>9.61 ± 0.47 (10)</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>30.51 ± 0.35 (31)</td>
<td>30.32 ± 0.24 (30)</td>
<td>27.95 ± 0.24 (28)</td>
<td>26.73 ± 0.20 (27)</td>
</tr>
<tr>
<td>Leucine</td>
<td>39.62 ± 0.47 (40)</td>
<td>40.69 ± 0.23 (41)</td>
<td>39.17 ± 0.41 (39)</td>
<td>39.94 ± 0.20 (40)</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>26.51 ± 0.17 (27)</td>
<td>26.33 ± 0.19 (26)</td>
<td>24.39 ± 0.16 (24)</td>
<td>22.75 ± 0.29 (23)</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>19.78 ± 0.20 (19)</td>
<td>18.99 ± 0.17 (19)</td>
<td>17.53 ± 0.37 (18)</td>
<td>16.73 ± 0.38 (17)</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>5.89 ± 0.25 (6)</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

**TOTAL** | 480                   | 480                   | 480                   | 480                   |

**Light Chain** | Residues/mol | **Heavy Chain** | Residues/mol |
<table>
<thead>
<tr>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>4.62 ± 0.28 (5)</td>
<td>Histidine</td>
<td>3.57 ± 0.16 (4)</td>
</tr>
<tr>
<td>Arginine</td>
<td>0 (0)</td>
<td>Aspartic acid</td>
<td>10.55 ± 0.04 (10)</td>
</tr>
<tr>
<td>Serine</td>
<td>9.69 ± 0.12 (10)</td>
<td>Threonine</td>
<td>19.10 ± 0.17 (22)</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>5.66 ± 0.16 (6)</td>
<td>Proline</td>
<td>22.20 ± 0.17 (22)</td>
</tr>
<tr>
<td>Glycine</td>
<td>10.71 ± 0.16 (11)</td>
<td>Alanine</td>
<td>20.07 ± 0.12 (20)</td>
</tr>
<tr>
<td>Alanine</td>
<td>3.35 ± 0.07 (3)</td>
<td>Valine</td>
<td>5.95 ± 0.11 (6)</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>7.16 ± 0.16 (7)</td>
<td>Methionine</td>
<td>30.46 ± 0.17 (10)</td>
</tr>
<tr>
<td>Leucine</td>
<td>1.02 ± 0.03 (1)</td>
<td>Tyrosine</td>
<td>32.72 ± 0.48 (13)</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>7.88 ± 0.13 (6)</td>
<td>Phenylalanine</td>
<td>16.66 ± 0.25 (17)</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>3.84 ± 0.13 (4)</td>
<td>Tryptophan</td>
<td>11.14 ± 0.15 (13)</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td>2.05 ± 0.07 (2)</td>
<td><strong>TOTAL</strong></td>
<td>4.88 ± 0.11 (5)</td>
</tr>
</tbody>
</table>

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*The data are the average and standard deviation of five to ten analyses.*
Cathepsin D isozymes from porcine spleens. Large scale purification and polypeptide chain arrangements.  
J S Huang, S S Huang and J Tang  