Multiple Forms of Cathepsin D from Bovine Uterus*

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SUMMARY

Preparations of cathepsin D from the bovine uterus contain at least 12 distinct forms of enzyme which digest hemoglobin at pH 3.2. Ten of these forms (2 through 11) have been purified by DEAE-Sephadex chromatography and disc electrophoresis. Six major forms (4, 5, 6, 7, 8, and 12) account for 94% of the total cathepsin D activity of the uterus. These six forms have been unequivocally identified as cathepsin D on the basis of their specificity in cleaving the B chain of insulin. Forms 2 and 3 show pH optima at pH 3.2 and are 50% inhibited by 0.001 M dithiothreitol in agreement with the properties of cathepsin D. Forms 1, 9, 10, and 11 are present only in trace amounts; they have approximately the same molecular weight as cathepsin D and digest hemoglobin at acid pH. Forms 2 through 8 have also been resolved by isoelectric focusing; their isoelectric points range from 5.0 to 7.2. Disc electrophoresis in the presence of sodium dodecyl sulfate and mercaptoethanol gave a single band of molecular weight 40,000 to 42,000 for Forms 2, 3, and 4; Forms 5 through 9 gave two distinct bands corresponding to weights of 13,000 to 14,000 and 25,000 to 28,000. Equilibrium ultracentrifugation gave a weight average molecular weight of 40,000 to 42,000 for Forms 2, 3, and 4; Forms 5 through 9 gave two distinct bands corresponding to weights of 13,000 to 14,000 and 25,000 to 28,000. Equilibrium ultracentrifugation gave a weight average molecular weight of 40,000 for Form 4 and slightly lower weights for Forms 5 and 6. Form 4 was more stable in acid and urea than Form 5. Forms 4 through 7 all had the same $V_{max}$ and $K_m$ when hemoglobin was used as substrate. This evidence suggests that Form 4 is the primary form of cathepsin D, that it consists of a single polypeptide chain of molecular weight 40,000, and that this chain is cleaved into inactive fragments by pancreatic trypsin inhibitor without loss of activity into fragments of 14,000 to 14,000 and 25,000 to 28,000.

As yet, no one knows the underlying structural basis of the multiplicity of forms. Evidence is presented in the present study that the enzyme may consist of a single polypeptide chain which undergoes limited cleavage without loss of activity. The results of the present study have been described briefly in preliminary reports (9, 12, 13).

EXPERIMENTAL PROCEDURE

Initial Purification of Enzyme—Protein assay methods, the assay of proteolytic activity on acid-denatured hemoglobin, the definition of enzyme units, and the method of purifying cathepsin D from bovine spleen have all been described recently by Woessner and Shamberger (7). In the present work a small modification has been introduced in the final step of purification. In this last step the enzyme is passed through a DEAE-Sephadex column equilibrated with 0.005 M phosphate buffer, pH 7.0. Prolonged washing with the same buffer brings about 85% of the enzyme out of the column and results in a 3-fold purification. Early fractions from this column are normally discarded because they contain considerable protein but only slight activity. However, in the present study, these early fractions were not dis-
carded since they contain several minor forms of cathepsin D. Therefore, all the 0.005 M phosphate buffer washings are combined and designated as Fraction C-I (C is a mnemonic for cow).

About 15% of the activity applied to the DEAE-Sephadex column was not eluted at low ionic strength. Elution with 500 ml of 0.2 M phosphate buffer, pH 7, brought out this fraction which was designated C-II. A great deal of protein impurities also emerged under these conditions, so that the specific activity of cathepsin was low (12 units per mg of protein). Fractions C-I and C-II were concentrated by lyophilization or by ultrafiltration through UM-10 membranes (Amicon, Lexington, Mass.). These two fractions contained essentially all of the activity that was present before the DEAE-Sephadex step and they served as the starting material for subsequent purifications.

Isolation of Multiple Enzyme Forms—Fractions C-I and C-II were dialyzed to equilibrium against 0.001 M phosphate buffer, pH 8.4, and chromatographed on DEAE-Sephadex columns as described in Fig. 1 (see below). Active fractions were pooled, concentrated by ultrafiltration on Amicon UM-10 membranes, and examined by disc electrophoresis for their enzyme form content.

Electrophoresis on basic gels was by the method of Davis (14) and Ornstein (15), using the standard 7.5% acrylamide gel which stacks at pH 8.9 and runs at pH 9.5; 200 to 400 µg of protein were applied in Sephadex G-25 suspension or in 10% sucrose above the spacer gel. Gels were stained with 0.5% Amido black in 7% acetic acid or with Coomassie blue by the method of Chrambach, Reisfeld, and Wyckoff (16). Duplicate unstained gels were sliced into uniform sections, macerated in 0.005 M phosphate buffer, pH 7.9, and assayed for protease activity by the direct addition of hemoglobin substrate. About 80% of the activity can be recovered in this way.

Not one of the chromatographic fractions consisted of a single enzyme form; all contained at least two bands. Moreover, the several bands that were present were usually quite close together. Therefore, the final separation of the individual forms was accomplished by extended disc electrophoresis on gel columns (4 × 60 mm). A concentrated chromatographic peak was distributed to as many as 36 gel columns, applying 300 µg of protein per column. Twelve columns were run together in the No. 1004 apparatus (Buchler Instruments, Fort Lee, N. J.) for sufficient time to bring the active bands into the lower 20 mm of the column. One gel was stained and the other 11 were immediately cut into 34 uniform slices. The active slices, as revealed by the stained gel, were combined and extracted in buffer as above. Purity was checked by disc electrophoresis and by isoelectric focusing in polyacrylamide gels. In these studies acrylamide electrophoresis reagents low in ultraviolet-absorbing impurities (Bio-Rad Laboratories, Richmond, Calif.) were used to permit protein estimations on the extracted enzyme.

Isoelectric Focusing—The isoelectric points of the enzyme forms were determined by isoelectric focusing on polyacrylamide gels by the procedure of Wrigley (17) using 6 × 100 mm, 7.5% gel columns. Ampholine carrier ampholyte (LKB Instruments, Rockville, Md.) in the range pH 5 to 8 was used. The enzyme solution was mixed with the polyacrylamide-ampholyte solution and photo polymerized in the cold. Focusing was carried out in the cold with a current of 1 mA per tube. The gels were cut into 2 mm sections which were then extracted in 1 ml of H2O. The extracts were tested for pH and enzyme activity. Trichloroacetic acid-fixed control gels were stained with Coomassie blue by the method of Riley and Coleman (18). Blank gels without enzyme were also analyzed.

Properties of Multiple Forms—The specificity of the major forms 4 through 8 was tested by the use of the B chain of insulin as a substrate of known peptide sequence. The conditions of digestion, the method of fingerprinting and the identification of the resultant peptides have been described previously (7). Identity of the peptides in the present study has been based on the comparison of the fingerprint pattern for each form with the pattern for the whole enzyme preparation.

Kinetic properties of the forms were determined at pH 3.2 using final hemoglobin concentrations of 0.4, 1.0, 2.0, 4.0, 13.33, 20.0, and 26.67 mg of hemoglobin per ml. K m and V max were determined by use of the computer program of Hanson, Ling, and Hlavir (19) which is based on the statistical techniques of hyperbola fitting of Bliss and James (20).

Equilibrium molecular weights of the major forms were determined by the monoseous depletion method of Yphantis (21). Enzyme preparations in 0.001 M phosphate buffer, pH 8.8, were passed through very small Sephadex G-100 columns (3 ml) to remove impurities extracted from the polyacrylamide gels. Enzyme concentrations of 0.2 to 1.0 mg of protein per ml were analyzed in the Yphantis six-channel centerpiece in the Spinco model E analytical ultracentrifuge. Photographs of the Rayleigh interference fringe patterns were measured with the Nikon 6-C microcomputer. The results were calculated using the Fortran computer program of Small and Resnick (22) as modified by us for the IBM 360/65 computer.

Analyses of the NH2-terminal amino acids were by the dansylation technique of Gray and Hartley (23). The derivatives were chromatographed on polyamide sheets by the method of Woods and Wang (24).

Separation of Subunits—The enzyme forms were dissociated in 1% recrystallized sodium dodecyl sulfate and 1% mercaptoethanol for 2 to 16 hours at 37° and then subjected to electrophoresis in polyacrylamide gels according to the methods of Weber and Osborn (25). Marker proteins for molecular weight estimations were purchased from Mann Research Corporation, N.Y. Quantitation of the subunits was accomplished by recording the absorption of the stained gels at 550 nm in the Gilford gel scanning spectrophotometer (Gilford Instrument Labs, Oberlin, Ohio). Parallel experiments were also performed on enzyme forms denatured in urea and mercaptoethanol or dithiothreitol (0.001 M). Electrophoresis in 8 M urea was run at pH 9.5 by the procedure of Rheeberg and Rueckert (26) and at pH 4 by the method of Parish and Marchalonis (27).

Results

Separation of Individual Enzyme Forms—The starting materials for the separation of the multiple forms of cathepsin D were the two Fractions C-I and C-II described under "Experimental Procedure." The results of the chromatography of these two fractions on DEAE-Sephadex are shown in Fig. 1. Fraction C-I showed a broad region of activity with four or five peaks discernible (Fig. 1A). The eight numbered forms were resolved by disc electrophoresis performed on samples from different regions of the elution pattern. Fraction C-II gave a pattern which overlapped that of C-I (Fig. 1B). This is surprising since in the original preparation of C-II the column had been exhaustively washed after the elution of C-I and before the elution of C-II at higher ionic strength (7). However, there seems to be little
doubt of the correspondence of the forms since they behave similarly on DEAE-Sephadex and on disc electrophoresis. The chief difference is that Fraction C-II lacks the more positively charged forms and has additional negative Forms 9 to 11. If one returns to the stage of purification prior to the separation of C-I and C-II (7), one sees that the eluate from G-100 Sephadex chromatography gives a disc electrophoresis pattern as shown in Fig. 2. This pattern contains all 11 forms later recovered from C-I and C-II. Fig. 2 illustrates the location of these forms which were assigned arbitrary numbers, starting with the least negatively charged form. The relative proportions of these active forms in the C-I and C-II fractions were estimated by prolonged electrophoresis of these fractions until they moved to the bottom third of 9-cm gel columns. This was followed by assay of the enzyme in thin serial slices of the gel. In the original preparation (sum of C-I plus C-II), Forms 2 through 10 are present in the ratio of 1.5:2.5:3.5:4.5:9:10:11:14:11:3:1.5:0.5 (Fig. 2). Forms 1 and 11 are present only as traces. Form 12 will be shown below to be included in Form 5. The four major forms, 4 through 7, account for over 90% of the activity, and most of our studies have had to center on these four forms. The major forms were all obtained from Fraction C-I. C-II was used only for the preparation of the minor Forms 8 to 11.

Complete separation of the forms was finally achieved by following the gradient chromatography with extended disc electrophoresis of the concentrated peak fractions. Each fraction was run for the length of time needed to bring its predominant form into the lower third of long gel columns. This increased the distance between the bands and allowed exclusion of the overlapping areas between them. The purest preparations were obtained by cutting out only the central section of each band and eluting in 0.005 M phosphate buffer, pH 7.0. In this way, pure samples of Forms 2 through 10 were prepared (Fig. 3). Obviously this method severely restricted the amounts of enzyme available for further study. Isoelectric focusing of the DEAE-Sephadex peaks in sucrose density gradients also produced separation of most of the multiple forms. However, isolation by extended disc electrophoresis proved more useful because of the difficulties encountered in removing the ampholytes introduced in the focusing method.

The starting material had a specific activity of about 70 units per mg of protein. The purified Forms 4, 5, 6, and 7 each had activities of 130 to 150 units per mg of protein. The minor forms were not generally obtained in high enough yield to provide reliable protein measurements. A specific activity of 150 corresponds to about a 2000-fold purification from the crude uterine extracts. Approximate estimations indicate that 100 units by our assay method correspond to 21 units of Press, Porter, and Cohn (8) and 360 units of Barrett (10). Therefore, the specific activity of the uterus cathepsin D may be 50% higher than that of the spleen enzyme and the human and chicken liver enzyme.

**Isoelectric Points**—The isoelectric points of the different forms were estimated by isoelectric focusing in acrylamide gel columns containing added ampholyte (Fig. 4). When the gels were cut into uniform thin sections, the pH of the sections was found to be an essentially linear function of the distance along the gel. However, there were uncertainties in the pH estimation due to the dilution resulting from extracting the gel slices and from the presence of small amounts of buffer in the enzyme samples. For this reason, the B and C forms of carbonic anhydrase (gift of Dr. P. H. Whitney, University of Miami) were used as markers.

**Fig. 1.** Column chromatography of Fractions C-I and C-II on DEAE-Sephadex. Five milliliters of C-I (Fig. 1A) or C-II (Fig. 1B) containing 300 units of enzyme activity were applied to columns (1.2 X 35 cm) of DEAE-Sephadex A-25. Both columns and protein solutions were equilibrated with 0.001 M sodium phosphate buffer, pH 8.4. The gradient elution employed phosphate buffer at pH 8.4 and increasing concentrations as shown by --- and upper right-hand scales. Protein was monitored at 280 nm (---, and lower right-hand scales). Cathepsin activity was measured against hemoglobin at pH 3.2 (---, left-hand scale). The circled numbers indicate the most prominent form found in each region by disc electrophoresis.
FIG. 3. Disc electrophoresis of the isolated multiple forms of cathepsin D. Illustrated from left to right are Forms 2 to 11, previously purified by DEAE-chromatography and then by cutting out from disc electrophoresis gels. Form 12 is not resolved from Form 5. Form 1 was not purified. Each form was subjected to electrophoresis under the same conditions except that varying amounts of enzymes were used.

since their isoelectric points had been determined recently by gradient isoelectric focusing (28). This introduced a correction factor of approximately +0.3 pH units. The corrected isoelectric points range from pH 5.5 for Form 8 to pH 7.2 for Form 2. During this study it was found that Form 5 could be resolved into two bands, the more negative of which has been named Form 12.

Identification of Forms as Cathepsin D— The main criterion for proving that an enzyme is cathepsin D is its specificity in cleaving the B chain of insulin. When Fraction C-I and purified enzyme Forms 4, 5, 6, 7, and 8 were tested for their specificity of action on the B chain of insulin, they all produced fingerprint patterns of B chain peptides which corresponded almost exactly to that found previously for the C-I fraction by Woessner and Shamberger (7). In every case there were major points of cleavage at Leu11-Tyr12, Phe7-Phe8, Phe6-Tyr7 and slower cleavage at Glu12-Ala13, Ala11-Leu12, and Tyr17-Leu18. All forms except 7 showed a split at Phe1-Val1. This minor difference is not felt to be significant since cleavage of this bond is very slow and might fail to occur if there were small differences in enzyme levels or in the pH adjustment.

A study of the pH optimum was made for Forms 2 through 8 using hemoglobin as substrate. The optimum was at pH 3.2 for forms 2, 3, and 5 and at pH 3.4 for Forms 4, 6, 7, and 8.

It may be concluded that Forms 4, 5, 6, 7, and 8 (94% of the total activity) meet all criteria for classification as cathepsin D. Forms 2 and 3 show the proper pH optima and also show 50% inhibition by 0.001 M dithiothreitol, in agreement with the properties of cathepsin D (7). The remaining forms, 1, 9, 10, and 11, have not been characterized and the only thing that can be said is that they emerge from Sephadex G-100 together with the other forms and that they digest hemoglobin at acid pH.

Structural Differences Among Major Forms—Those forms which could be obtained in sufficient amount were subjected to electrophoresis in the presence of sodium dodecyl sulfate and mercaptoethanol. As shown in Fig. 5, Forms 2, 3, and 4 moved as single bands with molecular weights around 40,000 to 42,000 and did not show any dissociation into subunits. Forms 5, 6, 7, 8, and 9 all showed dissociation into two bands with mobilities corresponding to 25,000 to 28,000 and 13,000 to 14,000, respectively. The mobilities of the various forms have been plotted along with the mobilities and weights of markers in Fig. 6. That the larger fragments of Forms 5, 6, 7, and 8 have different mobilities is shown by coelectrophoresis in Fig. 5 (e and f). The higher numbered forms always show a band corresponding to a weight of about 40,000. It is not known whether this represents incompletely dissociated enzyme, reaggregated protein, or small
FIG. 5. Electrophoresis in sodium dodecyl sulfate-polyacrylamide gels according to the methods of Weber and Osborn (23). The three groups of gels were run under slightly different conditions. The three lines indicate (from top to bottom) the position of the three markers: ovalbumin, 43,000; chymotrypsinogen, 24,000; and myoglobin, 17,000. a, Form 2 (seen as single faint band above the ovalbumin position); b, Form 3; c, Form 4; d, Form 5; e, Forms 5 and 6 combined plus myoglobin marker; f, Form 6; g, the three protein markers; h, Forms 6 and 7 combined; i, Form 7; j, Form 8; k, Forms 9 and 10 combined.

Extensive efforts were made to ensure that dissociation was as complete as possible. Studies were done with all of these forms in sodium dodecyl sulfate and in 8 M urea both with and without mercaptoethanol or dithiothreitol. In no case did Forms 2, 3, and 4 dissociate nor did the other forms show more than two additional bands. Forms 5 and higher do not have their subunits held together by S-S bridges, since they dissociate without any treatment by reducing agents.

Densitometry tracings were run on the sodium dodecyl sulfate gels of Forms 5, 6, 7, and 8. In each case the integrated area under the curve for the small component was almost exactly half of the area under the curve for the larger component. Assuming equal staining per unit length of peptide, this would indicate a 1:1 mole ratio of large to small fragment.

End group analyses were made by the dansylation of Forms 4, 5, 6, and 7. In each case glycine was the common NH₂-terminal amino acid. Form 4 did not show any other terminal residue. Forms 5, 6, and 7 each showed several faint spots, but none of these was comparable in intensity to the glycine spot. Either the point of internal cleavage involved a second glycine or else there were several points of cleavage so that no one end group predominated. This point cannot be resolved until the enzyme forms can be prepared on a larger scale and the individual subunits purified. It should be noted that the NH₂-terminal glycine was also reported by Press et al. (8) for two forms of bovine spleen cathepsin D.

Cathepsin D was found to have a weight of about 43,000 by
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TABLE I

Weight average molecular weights of cathepsin D obtained by equilibrium ultracentrifugation

<table>
<thead>
<tr>
<th>Enzyme form</th>
<th>No. of samples</th>
<th>$M_w \times 10^{-2}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>5</td>
<td>30.6 ± 2.8</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>37.7 ± 2.1</td>
</tr>
<tr>
<td>6, 7</td>
<td>3</td>
<td>36.9 ± 3.2</td>
</tr>
</tbody>
</table>

This refers to the number of separate and distinct preparations of each form.

Mean ± standard deviation of the whole cell average molecular weights.

These two forms were not resolved.

Fig. 8. Stability of cathepsin D to urea and pH. (●, Form 4; ○, Form 5; ×, Form 6.) The enzyme was incubated for 1 hour, 24°C in urea containing 0.005 M citrate buffer, pH 3.2. An equal volume of 2% hemoglobin at pH 3.2 was then added, and incubation was continued at 37°C as in the usual assay. The result in the absence of urea was taken as 100% activity. The pH stability was studied by incubating the enzyme at 37°C in 0.05 M sodium citrate buffers of various pH values for 2 hours. The pH was then restored to 3.2 and the enzyme was assayed with hemoglobin. Unincubated enzyme provided the 100% activity measurement.

chromatography on Sephadex G-100 (7). Sodium dodecyl sulfate electrophoresis gave weights ranging from 39,000 to 42,000. Independent measurements were made by equilibrium ultracentrifugation. Fig. 7 shows that a typical enzyme preparation is fairly homogeneous; however, a slight upward curvature indicates the presence of some heterogeneity. The curvature was a little more pronounced for Forms 5 and 6, suggesting a possible dissociation or degradation during the run. The average of a number of determinations (Table I) gave weight average molecular weights ranging from 37,000 to 40,000. These are slightly lower than the molecular sieve weights and are closer to the sodium dodecyl sulfate weights. Possibly there is some asymmetry of the molecule leading to higher weight estimations in Sephadex G-100.

The stability to pH and urea was studied for several of the forms (Fig. 8). As might be expected, the single chain Form 4 was more stable to acid and urea than the dissociable Forms 5 and 6; that is, it required a lower pH or a higher urea concentration to cause an activity loss in Form 4 compared with 5 and 6.

Finally, the kinetic parameters $K_m$ and $V_{max}$ were determined for Forms 4, 5, 6, and 7 (Table II). The hemoglobin assay is not very satisfactory for this purpose due to the typical large scatter in the results. However, it can be seen that there is no statistically significant difference in these two parameters for any of the four forms.

DISCUSSION

Cathepsin D of the bovine uterus has been separated into 12 different forms. Forms 1 through 11 have been numbered in order of increasing negative charge. Form 12 overlies Form 5 on disc electrophoresis and has only recently been resolved from 5 by isoelectric focusing. No separate studies have been performed on Form 12, but since the catalytic properties of Forms 4, 5, 6, and 7 are the same, it is believed that 5 and 12 do not differ significantly in their catalytic properties. Of the 12 forms discussed here, six (4, 5, 6, 7, 8) constitute about 94% of the total activity. Rigorous proof of their identity as cathepsin D has been established on the basis of their specificity of digestion of the B chain of insulin. Various clues point to the other six forms as also being cathepsin D forms, but rigorous proof must await the isolation of larger quantities.

It is not certain that these 12 forms represent all of the forms present in the uterus. Disc electrophoresis could not be applied until after the dioxane step (7) by which time 40% of the total activity had been lost. However, since 12 forms did survive through all the purification steps, and since no one purification step involved losses of more than 20%, it seems unlikely that any major forms have been lost. On the other hand, there is no guarantee that the present 12 forms may not be further resolved. In this regard, a comparison with the results of Press et al. (8) is instructive. Our C-I fraction (Fig. 1A) produced an elution pattern strikingly similar to that obtained by Press et al. (8) for bovine spleen cathepsin D. They also found that under each peak or region of the curve, electrophoresis revealed several forms. However, they inclined toward considering each of the forms under one DEAE-cellulose peak distinct from the corresponding forms under an adjacent peak, even if both moved to the same place upon electrophoresis. Therefore, they count 10 forms in the region where we count 6 forms (4, 5, 12, 6, 7, 8). If the two organs are similar, they have either counted some forms twice or we have failed to resolve some of our forms.

The multiple forms in the cow uterus may also be compared...
with these in human and chicken liver as reported by Barrett (10). Barrett found fewer forms, only three in each case. These forms all moved with ovalbumin on Sephadex G-100, as indeed, do the uterine forms. The isoelectric points of the human forms were 5.7, 6.0, and 6.5 (10). This places them in a position corresponding approximately to our Forms 6, 7, and 8. This identification is substantiated by personal communication from Barrett indicating that his forms all dissociate in sodium dodecyl sulfate to pieces similar to those reported here.

This leads directly to the most important question. "What is the structural or chemical basis of the multiple forms of cathepsin D found in the bovine uterus?" We would like to propose as a working hypothesis that the major forms all arise from a single chain of molecular weight approximately 41,000. It is suggested that Form 4 is the prototype form; it is also the most abundant species (32%) since Form 5 (24%) is found by isoelectric focusing to be composed almost equally of Forms 5 and 12. Of course, it cannot be excluded that Forms 2 and 3 are also prototype chains since they, too, do not dissociate in sodium dodecyl sulfate. It will be a difficult problem to isolate sufficient of the low numbered forms to explore the reasons for their differences from Form 4. Since there are no subunits in Forms 2 and 3, it seems likely that these forms differ either in the loss of a small peptide fragment or in the blocking or addition of charged side groups.

It is next suggested that Form 4, or a closely similar species, undergoes chain seission which produces two unequal peptide chains with molecular weights of approximately 27,000 and 13,000. These two pieces are held together by noncovalent forces and retain full enzymatic activity. The close relation of Forms 5, 6, and 7 to Form 4 is suggested by the observation that all have the common end group glycine, the same specific activity (130 to 150 units per mg of protein), and the same kinetic constants. Cleavage of the chain does not seem to impair the catalytic function of the enzyme.

It is not clear whether cleavage of the chain involves only a single bond, or whether pieces of the chain are removed. Sodium dodecyl sulfate electrophoresis indicates decreasing weights of the larger fragment as one goes to higher numbered forms. However, it is possible that the sodium dodecyl sulfate has failed to completely dissemble the charge differences in the different forms, so that mobility cannot be precisely equated to molecular weight (cf. Reference 29). This question cannot be resolved until amino acid analyses and further end-group studies are made. If proteolytic cleavage is limited to a single bond, we must then invoke other explanations for the multiplicity of Forms from 5 through 11. There are not enough single chain forms to account for the forms with two peptide chains.

Consideration might also be given to the possibility that there are three subunits of weight 13,000. Quite strenuous efforts have failed to dissociate the single chains of Forms 2 to 4 or the larger subunits of 5 to 8. One would have to postulate the formation of a covalent bond of novel type linking the subunits in these cases. For the moment, it seems easier to find the origins of the multiplicity at some point other than in a subunit structure involving genetically different subunits.

Continuing with the hypothesis that there is a fundamental chain (or possibly several genetically distinct forms of the chain) and that this chain undergoes limited proteolysis without loss of catalytic function, one must ask if the multiple forms occur naturally or arise during the isolation procedures. The case of yeast hexokinase (30) is instructive in showing how multiple isoenzyme forms of an enzyme may arise during purification due to limited cleavage by proteases present in the starting material. Moreover, the many similarities between cathepsin D and pepsin suggest the possibility of autolysis digestion to smaller active fragments as in the case of pepsin (31). However, both Press et al. (8) and Barrett (10) showed that the multiple forms of cathepsin D could be detected in the original crude tissue homogenate. We have performed a similar experiment in which crude uterine extracts were applied directly to large scale disc electrophoresis gels (Buchler Poly-Prep apparatus). It was possible to detect the four major bands of enzyme activity at this stage and they were present in approximately the same proportions and position on the gel as found for the purified enzyme (Fig. 2). We have never observed any conversion of one form to another upon lyophilization, storage at 4° for 6 months, or 18-hour incubation at 25° at pH values ranging from 3 to 8. Prolongation or shortening of suspicious steps such as the citrate dialysis step early in the purification has not altered the final pattern of forms, nor has storage of the frozen uterine tissue for as long as three years. Incubation of pure Form 4 at various temperatures and at pH values from 3.5 to 8.5 has not resulted in the appearance of any other forms. All evidence points to the inability of the enzyme to form multiple forms by autolysis. Proteolysis, if it does occur, must be attributed to other tissue proteases and it most likely occurs in the living tissue.

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REFERENCES


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