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# Pituitary Multicatalytic Proteinase Complex. Specificity of Components and Aspects of Proteolytic Activity†

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*Received April 24, 1989; Revised Manuscript Received June 29, 1989*

ABSTRACT: The 700-kDa multicatalytic proteinase complex from bovine pituitaries separates in polyacrylamide gel electrophoresis under dissociating and reducing conditions into 11 components with molecular masses ranging from 21 to 32 kDa. No higher molecular mass components were detected. A rabbit polyclonal antibody raised against the complex recognizes five immunoreactive components. As reported previously, the complex exhibits three distinct proteolytic activities designated as chymotrypsin-like, trypsin-like, and peptidylglutamyl-peptide hydrolyzing activities. All three activities are rather rapidly inactivated by 3,4-dichloroisocoumarin, a general serine protease inhibitor, however, the pseudo-first-order rate constants of inactivation of the three components differ within a wide range, with the chymotrypsin-like activity being most sensitive to inhibition. The peptidylglutamyl-peptide hydrolyzing activity is greatly activated by low concentrations of sodium dodecyl sulfate and fatty acids and seems to constitute the main component responsible for degradation of protein substrates. In addition to cleaving bonds on the carboxyl side of glutamyl residues, this activity also cleaves, albeit at a slower rate, bonds on the carboxyl side of hydrophobic residues; however, the secondary specificity of this component is clearly different from the chymotrypsin-like activity. Heparin selectively activates the chymotrypsin-like activity. The complex cleaves rapidly both native and dephosphorylated $\beta$-casein in a reaction greatly accelerated by low concentrations of sodium dodecyl sulfate. The nature of proteolytic products, and also the rate of formation of acid-soluble, ninhydrin-reactive products, is different for the phosphorylated and dephosphorylated form of $\beta$-casein, indicating that the degree of phosphorylation influences the rate and pattern of proteolysis. Lysozyme, human serum albumin, and phosphorylase *b* are also degraded but at a slower rate. Irreversible inhibition of the chymotrypsin-like activity leads to a marked activation of the trypsin-like activity, indicating interactions between components of the complex.

Previous work in this laboratory led to the identification in bovine pituitaries of a high molecular mass protein (700 kDa) which exhibited three proteolytic activities toward synthetic substrates containing hydrophobic, basic, and acidic amino acid residues in the $P_1$ position (Wilk et al., 1979; Wilk & Orlowski, 1980, 1983). Evidence was presented that the protein represents a multicomponent complex of nonidentical subunits and that the three activities are associated with distinct components of the complex (Orlowski & Wilk, 1981; Wilk & Orlowski, 1983). This led us to propose the name "multicatalytic proteinase complex" (MPC)[1] in recognition that the protein apparently represents a multienzyme complex, reminiscent of other multienzyme complexes known to occur in cells (Orlowski & Wilk, 1981, 1988; Wilk & Orlowski, 1983). Subsequent work in many laboratories showed that the complex is widely distributed in animal tissues, that it is apparently a constant component of all eucaryotic cells, and that it is composed of monodisperse particles with a cylindrical or disk shape, as shown by electron microscopy (Ray & Harris, 1985, 1987; Kopp et al., 1986; Dahlman et al., 1988; Rivett, 1989). Recent reports indicate that the complex is identical with the previously observed 19S ribonucleoprotein particles present in all eucaryotic cells (Shelton et al., 1970) for which the name "prosome" was proposed (Schmid et al., 1984). It is now clear that the MPC constitutes a major extralysosomal proteolytic system; however, it has not yet been firmly established whether other functions attributed to prosomes, such as repression of mRNA translation (Schmid et al., 1984), pre-tRNA 5′ processing endonuclease activity (Castaño et al., 1986), and aminoacyl transferase activity (Shelton et al., 1970), are indeed an integral part of the complex, nor is it certain whether small cytoplasmic RNA is indeed a constant part of the complex (Arrigo et al., 1988; Falkenburg et al., 1988).

† This work was supported by Grant DK 25377 from the National Institutes of Health.

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[1] Abbreviations: Cbz, benzyloxycarbonyl; Boc, *tert*-butoxycarbonyl; Bz, benzoyl; 2NA, 2-naphthylamide; DFP, diisopropyl fluorophosphate; DTT, dithiothreitol; Glt, glutaryl; MPC, multicatalytic proteinase complex; PAGE polyacrylamide gel electrophoresis; PCMB, *p*-mercuribenzoate; pNA, *p*-nitroanilide; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid.

0006-2960/89/0428-9270$01.50/0 

It is now firmly established that the complex contains at least three distinct proteolytic activities each associated with a different component of the complex. There are, however, questions related to the mechanistic classification of the proteolytic activities, the specificity of its components, the differential effect of inhibitors and activators, and the proteolytic activity of the complex toward protein substrates.

We report here an improved procedure for the isolation of an apparently homogeneous MPC from bovine pituitaries, and data which indicate that all three of its proteolytic components belong to the class of serine proteases. The results of studies on the effect of inhibitors and activators on the three proteolytic activities of the complex, the specificity of its components, and the pattern of degradation of several protein substrates are also presented.

## Materials and Methods

*Materials.* Frozen bovine pituitaries were obtained from Pel Freeze Inc. (Rogers, AR). Substrates were synthesized as described previously (Wilk et al., 1979; Wilk & Orlowski, 1980, 1983; Orlowski et al., 1983). The isocoumarin inhibitors were kindly provided by Dr. James Powers (Georgia Institute of Technology, Atlanta, GA). Poly(L-lysine) (MW 1500–8000) was obtained from United States Biochemical Corp. (Cleveland, OH). Phosphorylase *b* from rabbit muscle (twice crystallized), β-casein, and dephosphorylated β-casein were obtained from Sigma Chemical Co. (St. Louis, MO). Both casein preparations were obtained from bovine milk. Dephosphorylated β-casein was prepared enzymatically and as stated by the manufacturer was at least 80% dephosphorylated. Lysozyme and human serum albumin were obtained from Worthington Biochemical Corp. (Freehold, NJ). Ultrogel-AcA 22 was obtained from IBF Biotechnics Inc. (Savage, MD). Other proteins used as molecular weight standards as well as general reagents were obtained from Sigma Chemical Co.

*Determination of Enzyme Activity.* The chymotrypsin-like and peptidylglutamyl-peptide hydrolyzing activities of the complex were determined with Cbz-Gly-Gly-Leu-pNA and Cbz-Leu-Leu-Glu-2NA, respectively, as described previously (Wilk & Orlowski, 1980, 1983). The trypsin-like activity was determined with either Cbz-D-Ala-Leu-Arg-2NA or Bz-Leu-Met-Arg-pNA, using the same methods. Measurements are based on the determination of the aromatic amines released from the substrates using a diazotization procedure. Determination of activity toward other synthetic substrates is based on the same principle. Activity is expressed in units, 1 unit being defined as the amount of enzyme that liberates 1 μmol of product/h. Specific activity is expressed in units per milligram of protein.

Proteolytic activity was determined with β-casein, dephosphorylated β-casein, lysozyme, human serum albumin, and phosphorylase *b*. Proteolysis was measured by determining the release of acid-soluble peptides from the protein as expressed by an increase in ninhydrin-reactive products remaining in solution after precipitation of the protein substrates with trichloroacetic acid (TCA). Ninhydrin-reacting products were determined by using the procedure essentially as described by Rosen (1967). Reaction mixtures (final volume 150 μL) contained Tris-HCl buffer (0.01 M, pH 8.0), 50 μL of enzyme (about 10 μg of protein after step 7 of the purification procedure; see Table I), and 50 μL of a 0.3% β-casein solution in 0.01 M Tris-HCl buffer. In all experiments with proteolytic degradation, the amount of protein was identical (150 μg) in each incubation mixture. Incubations were carried out at 37 °C and terminated after various time intervals by addition of 50 μL of a 10% solution of TCA. Controls without incubation containing all components of the incubation mixture, and also controls in which either the enzyme or the substrate was incubated separately and combined before addition of trichloroacetic acid, were also prepared. The precipitated protein was removed by centrifugation. One hundred microliters of the supernatants was mixed with 200 μL of a potassium cyanide/sodium acetate buffer solution, prepared by diluting 2 mL of a 0.01 M KCN solution into 200 mL of 4 M sodium acetate buffer (pH 5.2). After addition of 100 μL of a 3% solution of ninhydrin in methyl cellosolve, the tubes were covered with glass marbles and placed in a boiling water bath for 10 min. The tubes were then cooled and diluted with 1 mL of a 1:1 mixture of 2-propanol/water. The absorbance of the ninhydrin-reacting material was determined at 570 nm, and the amount of degradation products was determined from a standard curve prepared under the same conditions with various concentrations of leucine. Activity is expressed as the number of nanomoles of leucine equivalents released. Specific activity is expressed in leucine equivalents per milligram of protein per hour.

Polyacrylamide gel electrophoresis (PAGE) under denaturing conditions in the presence of sodium dodecyl sulfate (SDS) was also used to visualize the polypeptide degradation products derived from proteolysis of protein substrates. Aliquots of reaction mixtures (20 μL) containing 20 μg of protein and about 1.1 μg of the complex were withdrawn after various incubation times, and after addition of 2.5 μL of a 10% solution of SDS and 2-mercaptoethanol to a final concentration of 5%, and heating at 70 °C for 10 min, they were subjected to PAGE (see below).

*Purification of the Enzyme.* All steps were carried out at 4 °C. The purification procedure is a modification of that previously described (Wilk & Orlowski, 1979, 1980, 1983). Fifty frozen bovine pituitaries (98 g) were washed free of blood and homogenized in 5 volumes of 0.01 M Tris-HCl buffer, pH 7.5. The homogenate was centrifuged at 27000*g* for 15 min, and the supernatant was collected. The precipitate was washed with 300 mL of the same buffer and centrifuged as above. The combined supernatants were treated with solid ammonium sulfate (24.3 g/100 mL) and centrifuged as above. The precipiate was discarded, and the supernatant was treated with additional ammonium sulfate (13.2 g/100 mL) and centrifuged. The pellet was dissolved in 15 mL of a Tris–EDTA buffer (0.01 M, pH 7.34) and dialyzed overnight against 7 L of the same buffer. The enzyme was then applied to the top of a DEAE-Sephacel column (50 mL) equilibrated with the same buffer, and washed with 110 mL of buffer. Elution was carried out with a linear gradient established between 200 mL of 0.01 M Tris–EDTA (pH 7.34) and 200 mL of 0.3 M Tris–EDTA of the same pH. Fractions containing activity were applied to an Ultrogel AcA-22 column (5 × 50 cm) equilibrated with 0.01 M Tris–EDTA buffer (pH 8.3). The column was eluted at a rate of 40 mL/min, and active fractions were pooled and applied to the top of a DEAE-Sephacel column equilibrated with a 0.01 M Tris–EDTA buffer (pH 8.3). The enzyme was eluted from the column with a linear gradient established between 150 mL of the equilibrating buffer and 150 mL of a 0.4 M Tris–EDTA buffer, pH 8.3. Fractions containing activity were pooled, concentrated to about 20 mL, and applied the same day to the top of an Ultrogel-AcA 22 column (5 × 50 cm) equilibrated with a 0.01 M Tris–EDTA buffer (pH 7.5), and eluted with the same buffer at a rate of 40 mL/h. Fractions of about 10 mL were collected and tested for activity and protein, by the

Table I: Summary of Purification of the Multicatalytic Protease Complex from Bovine Pituitaries[a]

| purification step | volume (mL) | protein (mg/mL) | activity units/mL | activity total | sp act. (units/mg) | recovery (%) | purification (*x*-fold) |
|---|---|---|---|---|---|---|---|
| (1) homogenate | 620 | 24.2 | 0.167 | 104 | 0.0069 | 100 | 0 |
| (2) supernatant | 815 | 5.29 | 0.102 | 83.1 | 0.019 | 80 | 2.8 |
| (3) ammonium sulfate fractionation | 26.5 | 21.8 | 1.46 | 38.7 | 0.067 | 37 | 9.7 |
| (4) DEAE-Sephacel chromatography (pH 7.34) | 100 | 0.52 | 0.583 | 58.3 | 1.13 | 56 | 164 |
| (5) Ultrogel chromatography (pH 8.3) | 114 | 0.116 | 0.474 | 54 | 4.08 | 52 | 591 |
| (6) DEAE-Sephacel chromatography (pH 8.3) | 64 | 0.125 | 0.542 | 34.7 | 4.34 | 33 | 629 |
| (7) second Ultrogel column chromatography | 70 | 0.040 | 0.66 | 36.4 | 16.5 | 35 | 2390 |

[a] Activity was determined with Cbz-Gly-Gly-Leu-pNA as the substrate (0.4 mM) as described under Materials and Methods and defined as the amount of enzyme that cleaves 1 μmol of the substrate per hour.

absorbance at 280 nm. The enzyme emerged as a single peak of activity after about 540 mL of the buffer had passed through the column. All experiments reported here were carried out on the enzyme after step 7 of the purification procedure.

*PAGE and Determination of Molecular Weights.* Disc PAGE was carried out under nondissociating conditions in a 0.05 M Tris-HCl buffer, pH 8.3; 10–20 μg of enzyme protein was layered on the top of 4% polyacrylamide gels, and a current of 4 mA per tube was applied for a time period necessary for the tracking dye to reach the bottom of the gel. Slab gel electrophoresis under dissociating conditions was carried out in a Hoeffer Scientific vertical slab gel electrophoresis apparatus. The thickness of analytical gels was 1.5 mm. A discontinuous buffer system essentially as described by Laemmli (1970) was used. A 12% polyacrylamide gel containing 0.1% SDS was used as the separating gel. All buffers contained 0.1% SDS. The isolated proteinase complex was treated with 1% SDS and 5% 2-mercaptoethanol, and two pairs of duplicate samples containing about 5 and 10 μg of protein were applied to the top of the gel after heating at 70 °C for 10 min. Molecular weight standards contained 2.5 μg of each protein (cytochrome *c*, trypsinogen, ovalbumin, and bovine serum albumin). The temperature of the gels was maintained during electrophoresis at 10 °C using a cooling coil. A current of 45 mA was applied, and electrophoresis was continued until the tracking dye migrated to a distance of 220–280 mm. Gels were then cut longitudinally in two parts; one half was stained for protein with Coomassie Brilliant Blue, and the other half was used for immunoblotting as described below.

*Raising of Antibodies and Immunoblotting.* The enzyme (0.3 mg) obtained after step 7 of the purification procedure was emulsified with Freund's complete adjuvant and injected intradermally at multiple sites on the back of a New Zealand white rabbit. The injection was repeated after 3 weeks with 0.4 mg of enzyme, and serum was obtained 7 days later from blood collected from the ear vein.

Electrophoretic transfer of proteins from SDS–polyacrylamide gels was carried out essentially as described (Towbin et al., 1979; Burnette, 1981) using a Trans-Blot electrophoretic transfer cell (Bio-Rad Laboratories, Richmond, CA). The first antibody was used in a 1:500 dilution. An affinity-purified goat anti-rabbit horseradish peroxidase conjugate (Bio-Rad) was used for visualization according to the manufacturer's recommendations.

*Inhibition Kinetics.* Isocoumarin inhibitors (10–30 μL) dissolved in dimethyl sulfoxide ($Me_2SO$) were preincubated with the enzyme in 0.01 M Tris–EDTA buffer, pH 7.5 (0.09–0.3 mL), at 23 °C. Aliquots of the preincubation mixture (10–50 μL) were transferred at various time intervals for determination of residual activity to reaction mixtures (final volume 0.25 mL) containing substrate (10 μL of a 10 mM solution in $Me_2SO$; final concentration 0.4 mM) and 0.05 M Tris-HCl buffer, pH 8.0 at 37 °C. The final concentration of $Me_2SO$ in the incubation mixtures was 5.2%. In experiments with DFP, the enzyme was preincubated with various concentrations of the inhibitor (23 μL of a solution of the inhibitor in 2-propanol) in a 0.05 M Tris-HCl buffer, pH 8.0 (final volume of 0.69 mL). Aliquots of the preincubation mixture were withdrawn at various time intervals and assayed for residual activity as described above. The pseudo-first-order inactivation rate constants ($k_{obs}$) were obtained from plots of ln ($v_i/v_0$) versus time. Correlation coefficients were generally better than 0.98.

## Results

A summary of the purification is given in Table I. An almost 2400-fold increase in specific activity was obtained with a yield exceeding 30%. The purification is facilitated by the high molecular mass of the enzyme (700 kDa) which favors the removal of lower molecular weight proteins by chromatography on Ultrogel columns. A rather significant increase in total enzyme recovery was seen in step 4. This may reflect an increase in activity due to the removal of significant amounts of protein and also removal of ammonium sulfate, since the presence of high salt concentrations inhibits enzyme activity. The specific activity of the isolated complex is about twice as high as that reported previously (Wilk & Orlowski, 1983). This increase in specific activity was apparently achieved by replacing the AcA-34 Ultrogel with AcA-22, by replacing the dialysis of the enzyme after step 4 of the purification with the molecular sieving chromatography step on Ultrogel AcA-22, and by minimizing the exposure of the enzyme to high ionic strength buffers after step 6 of the purification.

The isolated enzyme was homogeneous as judged by PAGE under nondissociating conditions and also by Ouchterlony (1958) double immunodiffusion experiments. A single sharp protein band was obtained when 20 μg of the enzyme was subjected to PAGE, and single precipitation lines with patterns of identity were obtained in double immunodiffusion experiments. These results were in agreement with those reported previously (Wilk & Orlowski, 1983). PAGE under dissociating conditions showed the presence of eight main components with molecular weights ranging from 21 000 to 32 000 (Figure 1). Components 3, 4, and 6, in increasing order of molecular weight, showed a tendency to separate into 2 bands with closely similar migration rates, indicating the presence of a total of 11 components. Only 5 of the 11 components reacted in immunoblots with the rabbit antiserum. Superimposition of the immunoreactive bands with the main Coomassie Brilliant Blue staining bands indicates that only the more intensely staining bands were visualized and that their location coincided with components 1, 3, 4, 6, and 8 in order of increasing molecular mass (Figure 1).

None of the components of the complex was inhibited by the antiserum in an anticatalytic immunoinhibition assay,

Table II: Inhibition of Components of the Multicatalytic Proteinase Complex by DFP and Isocoumarin Derivatives[a]

| inhibitor/component | chymotrypsin-like [I] (mM) | chymotrypsin-like $t_{1/2}$ (min) | chymotrypsin-like $k_{obs}/I$ ($s^{-1}$ $M^{-1}$) | peptidylglutamyl-peptide hydrolyzing [I] (mM) | peptidylglutamyl-peptide hydrolyzing $t_{1/2}$ (min) | peptidylglutamyl-peptide hydrolyzing $k_{obs}/I$ ($s^{-1}$ $M^{-1}$) | trypsin-like [I] (mM) | trypsin-like $t_{1/2}$ (min) | trypsin-like $k_{obs}/I$ ($s^{-1}$ $M^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|
| (1) DFP | 2.0 | 31.5 | 0.18 | 2.0 | NI | NI | 2.0 | NI | NI |
| (2) 3,4-dichloroisocoumarin | 0.002 | 45.5 | 131 | 0.006 | 78.2 | 28.3 | 0.015 | 64 | 12.1 |
| | 0.004 | 20.1 | 147 | 0.012 | 30.1 | 32.9 | 0.040 | 25.6 | 11.6 |
| (3) 7-amino-4-chloro-3-[3-(isothioureido)-propoxy]isocoumarin·HBr | 0.5 | 61.0 | 0.38 | 0.5 | 27.8 | 0.83 | 0.5 | 56.4 | 0.41 |
| (4) 4-chloro-7-guanidino-3-(2-phenylethoxy)-isocoumarin | 1.0 | 55.0 | 0.21 | 0.1 | 22.6 | 5.1 | 1.0 | 10.2 | 1.21 |
| (5) 4-chloro-3-ethoxy-7-nitroisocoumarin | 0.5 | NI[b] | NI | 0.5 | 44.0 | 0.53 | 0.5 | NI | NI |
| (6) 3-benzyloxy-4-chloroisocoumarin | 1.0 | NI | NI | 1.0 | NI | NI | 1.0 | NI | NI |

[a] Data are mean values from two to five separate determinations. For details of experimental design, see Materials and Methods. [b] No inhibition.

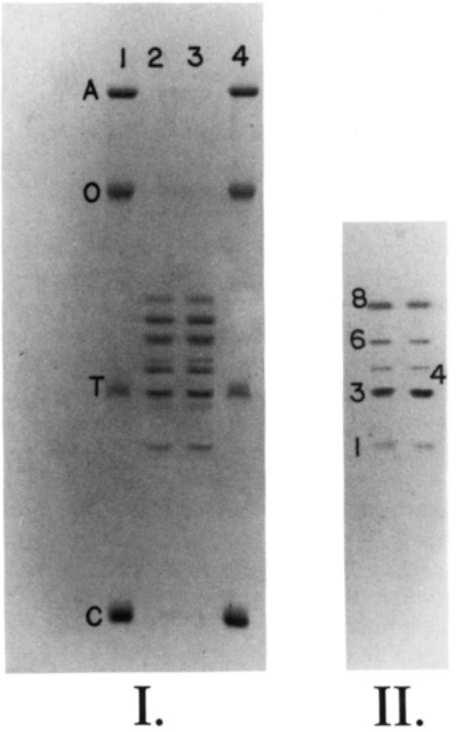


FIGURE 1: (I) Polyacrylamide gel electrophoresis of the enzyme under dissociating and reducing conditions. Lanes 1 and 4 contained 2.5 μg each of the molecular weight standards (A = bovine serum albumin; O = ovalbumin; T = trypsinogen; C = cytochrome *c*). Lanes 2 and 3 contained each 10 μg of the complex. (II) Western blots of the enzyme components shown in (I). The numbers indicate the correspondence of the immunoreactive bands with the main Coomassie Brilliant Blue staining bands (also see text for explanation).

indicating that the antiserum was binding to antigenic epitopes without interfering with the access of the small synthetic substrates to the active sites of the enzymes of the complex.

The effect of irreversible serine protease inhibitors on enzyme activity was studied by determining the pseudo-first-order inactivation rate constant of the enzyme after preincubation with DFP and several mechanism-based isocoumarin derivatives (Table II) which form acyl enzymes with the active-site serine residue in serine proteases (Harper et al., 1985; Harper & Powers, 1985; Kam et al., 1988). DFP at relatively high concentrations of 2–5 mM inactivated in a time- and concentration-dependent manner the chymotrypsin-like activity, but was without effect on the peptidylglutamyl-peptide and trypsin-like activities. The inactivation of the chymotrypsin-like activity with an inhibitor concentration of 2.0 mM proceeded rather slowly with a $t_{1/2}$ of about 30 min. The general serine protease inhibitor 3.4-dichloroisocoumarin, however, was the most potent inhibitor among the several isocoumarin derivatives tested. The three components of the complex, however, were inactivated at distinctly different rates. Thus, the chymotrypsin-like activity was inactivated most rapidly with $k_{obs}/I$ values in the range of 130–140 $s^{-1}$ $M^{-1}$, more than 10 times higher than those obtained for the trypsin-like activity. The inactivation of the peptidylglutamyl-peptide hydrolyzing activity proceeded at rates intermediate to those for the other two components. These data suggest that in spite of the low reactivity of the components with DFP, all three proteolytic activities apparently belong to the class of serine proteases. Inhibition studies with the other isocoumarin derivatives also support this conclusion. The results, however, obtained with the other isocoumarin derivatives indicate that the structure of the inhibitors greatly influences the rate of inactivation of the enzymes, probably by affecting access of the inhibitor to the active site. Thus, only 7-amino-4-chloro-3-[3-(isothioureido)propoxy]isocoumarin and 4-chloro-7-guanidino-3-(2-phenylethoxy)isocoumarin inhibited all three components of the complex, although at rates greatly lower than those obtained with 3,4-dichloroisocoumarin. Of the remaining isocoumarin derivatives, 4-chloro-3-ethoxy-7-nitroisocoumarin inhibited only the peptidylglutamyl-peptide hydrolyzing activity, whereas 3-benzyloxy-4-chloroisocoumarin was inactive.

Studies with the isocoumarin derivatives indicated the presence of an interaction or interdependence between the proteolytic components of the complex. Thus, while at low concentrations 3,4-dichloroisocoumarin progressively inhibited the chymotrypsin-like and peptidylglutamyl-peptide hydrolyzing activity, these concentrations caused a significant activation of the tryspin-like activity, a component that showed $k_{obs}/I$ values more than 10 times lower than those of the chymotrypsin-like activity (Table II). Preincubation of the complex with 2 μM 3,4-dichloroisocoumarin led to a progressive inactivation of the chymotrypsin-like component, and a more than 2-fold increase in the trypsin-like activity during a 30-min preincubation period (Table III). This trend was even more pronounced at an inhibitor concentration of 4 μM. At this concentration, both the chymotrypsin-like and peptidylglutamyl-peptide hydrolyzing activities were greatly inhibited, while the trypsin-like activity increased by more than 2.5-fold. These data indicate the possibility of a selective inactivation of the first two components with a concomitant activation of the latter, a condition making possible detailed selective studies of the specificity of the trypsin-like component. It should be noted that at higher concentrations of 3,4-dichloroisocoumarin (15–40 μM) the inactivation of the trypsin-like activity proceeded in a manner consistent with an apparent first-order reaction (Table II).

A series of low and high molecular weight compounds were active as inhibitors or selective activators of some of the proteolytic components. As reported previously (Wilk &

Table III: Effect of Low Concentrations of 3,4-Dichloroisocoumarin on the Activities of the Components of the Complex[a]

| substrate | preincubation time (min) | activity (units/mL) | relative activity | 3,4-dichloroisocoumarin concn ($\mu$M) |
|---|---|---|---|---|
| Z-Gly-Gly-Leu-pNA | 0 | 2.19 | 100 | 2 |
| | 30 | 1.37 | 63 | 2 |
| Z-Leu-Leu-Glu-2NA | 0 | 1.87 | 100 | 2 |
| | 30 | 2.03 | 108 | 2 |
| Bz-Leu-Met-Arg-pNA | 0 | 1.42 | 100 | 2 |
| | 10 | 2.25 | 158 | 2 |
| | 20 | 2.65 | 187 | 2 |
| | 30 | 3.02 | 213 | 2 |
| Z-Gly-Gly-Leu-pNA | 0 | 2.2 | 100 | 4 |
| | 75 | 0.32 | 15 | 4 |
| Z-Leu-Leu-Glu-2NA | 0 | 2.7 | 100 | 4 |
| | 75 | 1.45 | 54 | 4 |
| Bz-Leu-Met-Arg-pNA | 0 | 1.24 | 100 | 4 |
| | 35 | 2.96 | 239 | 4 |
| | 75 | 3.23 | 260 | 4 |

[a] Preincubations were at 23 °C and contained enzyme and inhibitor at a ratio (v/v) of 1:50. Aliquots of the mixtures (20–30 $\mu$L) were transferred to incubation mixtures (final volume 0.25 mL) at 37 °C for measurements of remaining activities. Activities were determined as described under Materials and Methods.

Table IV: Effect of Inhibitors and Activators on the Activity of the Components of the Complex[a]

| inhibitor | concn (mM) | relative activity: chymotrypsin-like | relative activity: peptidylglutamyl-peptide hydrolyzing | relative activity: trypsin-like |
|---|---|---|---|---|
| none | | 100 (203) | 100 (82) | 100 (177) |
| SDS | 0.69 | 96 | 839 | 5 |
| | 1.38 | 63 | 1490 | 3 |
| | 2.08 | 3 | 480 | 2 |
| | 2.77 | 0 | 8 | 0 |
| palmitic acid | 1.44 | 88 | 254 | 11 |
| | 4.32 | 133 | 369 | 1 |
| | 7.2 | 153 | 104 | 1 |
| linoleic acid | 0.71 | 86 | 414 | 66 |
| | 2.85 | 29 | 524 | 33 |
| | 7.13 | 8 | 201 | 6 |
| heparin | 2 units/mL | 114 | 76 | 88 |
| | 4 units/mL | 139 | 70 | 69 |
| | 10 units/mL | 104 | 51 | 26 |
| RNA | 0.065 mg/mL | 71 | 55 | 83 |
| | 0.26 mg/mL | 53 | 43 | 72 |
| | 0.65 mg/mL | 45 | 27 | 44 |
| ATP | 0.1 | 82 | 72 | 0 |
| | 1.0 | 65 | 29 | 61 |
| spermidine | 0.2 | 94 | 293 | 99 |
| | 1.0 | 85 | 140 | 79 |
| polylysine | 0.04 mg/mL | 52 | 18 | 85 |
| | 0.1 mg/mL | 54 | 21 | 83 |

[a] Activity of each of the components was determined as described under Materials and Methods. Values in parentheses represent nanomoles of substrate degraded per hour per milliliter of enzyme.

Orlowski, 1980), PCMB and *N*-ethylmaleimide inhibited all components of the complex, and leupeptin selectively inhibited the trypsin-like activity (data not shown). Yeast RNA and ATP inhibited all activities of the complex (Table IV), albeit to a different degree, while heparin, a polyanionic compound, selectively activated the chymotrypsin-like activity, while inhibiting the other two activities. At a concentration of 1.0 mM ATP, the peptidylglutamyl-peptide hyrolyzing activity was most strongly inhibited (71%), while the chymotrypsin-like and trypsin-like activities were inhibited by 35 and 39%, respectively. A similar selective pattern of inhibition was obtained with yeast RNA. At a concentration of 0.65%, the peptidylglutamyl-peptide hydrolyzing activity was inhibited by 73% while the other two activities were inhibited by 55%. Spermidine, a polyamine known to bind to DNA and to apparently stabilize the structure of DNA, ribosomes, and some viruses, activated the peptidylglutamyl-peptide hydrolyzing activities while inhibiting the other two activities. Polylysine reported previously to activate the multicatalytic proteinase from rat liver (Tanaka et al., 1986) inhibited the chymotrypsin-like and peptidylglutamyl-peptide hydrolyzing activities, while having little effect on the trypsin-like activity.

Table V: Activity of the Complex toward Several Synthetic Substrates and the Effect of Activators[a]

| substrate | relative activity: activity (units/mL) | relative activity: heparin (4 units/mL) | relative activity: SDS (0.04%) |
|---|---|---|---|
| (1) Cbz-Gly-Gly-Leu-pNA | 0.329 | 125 | 46 |
| (2) Cbz-(D)-Ala-Leu-Arg-2NA | 0.217 | 57 | 1 |
| (3) Cbz-Leu-Leu-Glu-2NA | 0.206 | 72 | 1480 |
| (4) Boc-Phe-Ala-Ala-Phe-pAB | 0.039 | 135 | 733 |
| (5) Bz-Gly-Ala-Ala-Phe-pAB | 0.049 | 123 | 191 |
| (6) Glt-Ala-Phe-Phe-pAB | 0.021 | 140 | 236 |
| (7) Bz-Gly-Phe-Ala-Ala-Phe-pAB | 0.076 | 113 | 729 |

[a] Activity was determined as described under Materials and Methods at a substrate concentration of 0.4 mM.

As reported previously (Wilk & Orlowski, 1983), the strongest activation was observed with low concentrations of SDS (15-fold). This activation was limited to the peptidylglutamyl-peptide hydrolyzing activity, while the other two activities were strongly inhibited. The activating effect of SDS was limited to a rather narrow concentration range and decreased precipitously at higher concentrations. At a concentration, however, of about 2 mM, the peptidylglutamyl-peptide hydrolyzing activity was still increased almost by 5-fold, while the other two activities were almost completely inhibited. A similar selective pattern of activation of the peptidylglutamyl-peptide hydrolyzing activity was observed with the fatty acids palmitic and linoleic acid. Although the activating effect of fatty acids was smaller than that of SDS, the concentration range at which the activation was seen was generally much wider, and the extent of inhibition of the other activities was lower. Indeed, palmitic acid showed some activating effect on the chymotrypsin-like activity.

The availability of selective activators and inhibitors of components of the complex provided a convenient approach to verifying the substrate selectivity of its components. Thus, in addition to the three substrates used to determine the three proteolytic components of the complex, we have studied the activity of the complex and the effect of heparin and SDS on four additional substrates having a hydrophobic group in the $P_1$ position (substrates 4–7, Table V). The activity of the complex toward these substrates was markedly lower than that against the other three substrates (substrates 1–3). As expected, heparin activated the chymotrypsin-like activity toward Cbz-Gly-Gly-Leu-pNA while inhibiting the trypsin-like and peptidylglutamyl-peptide hydrolyzing activity toward Cbz-(D)-Ala-Leu-Arg-2NA and Cbz-Leu-Leu-Glu-2NA, respectively. Similarly, SDS greatly activated the peptidylglutamyl-peptide hydrolyzing activity while the activities toward the Arg- and Leu-containing substrates (substrates 1 and 2) were strongly inhibited. Both heparin and SDS, however, activated the cleavage of substrates 4–7. These data are consistent with the interpretation that substrates 4–7, containing a Phe residue in the $P_1$ position, are apparently cleaved by both the chymotrypsin-like activity and the peptidylglutamyl-peptide hydrolyzing activity although at markedly slower rates. Consequently, it can be assumed that the peptidylglutamyl-peptide hydrolyzing activity also exhibits chy-

Table VI: Protein Degradation by the Multicatalytic Proteinase Complex[a]

| protein | activity (nmol of Leu equiv) for incubation time (min) | | | |
|---|---|---|---|---|
| | 60 | 120 | 180 | 240 |
| casein | 58.7 (264) | 136 (306) | 285 (429) | 338 (382) |
| casein + SDS | 518 (2330) | 1497 (3370) | | |
| casein dephosphorylated | 8.0 (36) | 85.4 (192) | 184 (276) | 309 (349) |
| casein, dephosphorylated, + SDS | 1140 (5140) | 1673 (3770) | | |
| lysozyme | | | | 45.4 (51.1) |
| lysozyme + SDS | | | | 104 (117) |
| human serum albumin | | | | 18.7 (21) |
| human serum albumin + SDS | | | | 165 (186) |
| phosphorylase *b* | | | | 40 (45) |
| phosphorylase *b* + SDS | | | | 157 (177) |

[a] Reaction mixtures (final volume 0.15 mL) contained protein substrate (150 μg), enzyme (11.1 μg of protein), and Tris-HCl buffer (0.01 M, pH 8.0). SDS where indicated was present at a final concentration of 0.04%. Values in parentheses represent specific activities expressed as nanomoles of leucine equivalents per milligram of protein per hour. The reaction was terminated by addition of 0.05 mL of 10% trichloroacetic acid (TCA), and the amount of TCA-soluble leucine equivalents was determined in an aliquot of the supernatant after removal of the protein precipitate by centrifugation.

motrypsin-like activity. This assumption is supported by the finding (data not shown) that inactivation of the chymotrypsin-like activity toward Cbz-Gly-Gly-Leu-pNA by preincubation of the complex with 5 mM DFP only partially inhibits the activity of the complex toward substrates 4 and 5 and that the remaining activity toward these substrates is strongly stimulated by the presence of 0.04% SDS. It is clear, however, from the effect of SDS (Table IV), that the Cbz-Leu-Leu-Glu-2NA hydrolyzing activity does not cleave Cbz-Gly-Gly-Leu-pNA and that, vice versa, the chymotrypsin-like activity does not cleave Cbz-Leu-Leu-Glu-2NA. Thus, the peptidylglutamyl-peptide hydrolyzing activity, while cleaving substrates with a hydrophobic residue in the $P_1$ position, shows a preference for substrates with a Glu residue in this position and also a preference for hydrophobic residues in the $P_2$ and $P_3$ positions (Leu residues in substrate 3, and Ala or Phe residues in substrates 4–7). It is possible that the Phe-containing peptides (substrates 4–7) contain structural determinants capable of interacting with the active site of both the chymotrypsin-like and peptidylglutamyl-peptide hydrolyzing activity, in contrast to substrates 1–3 each of which apparently interacts only with a single proteolytic component of the complex.

Several proteins were tested for degradation by the complex, both by following the release of acid-soluble ninhydrin-reacting material and also by following the degradation of the protein by PAGE. The results of representative experiments are shown in Table VI. Among the proteins tested, casein and dephosphorylated casein were degraded most rapidly. Lysozyme, human serum albumin, and phosphorylase *b* were degraded at a rate from 7 to almost 20 times slower. Examination of the time course of release of ninhydrin-reactive material showed that there was an initial lag period, after which the reaction markedly accelerated. This could be explained by the initial formation of higher molecular weight intermediates with a low ninhydrin reactivity, followed by degradation to lower molecular weight, ninhydrin-positive products. The results also show a clear difference in the progress of proteolytic degradation of casein and dephosphorylated casein. Thus, for example, after 60-min incubation, there was a 7-fold higher release of leucine equivalents from phosphorylated casein than from dephosphorylated casein. At longer incubation times, the rate of reaction of dephosphorylated casein increased so that after 4 h the amount of ninhydrin-reacting material was almost the same for both phosphorylated and dephosphorylated casein. This pattern of proteolysis could be explained by assuming that phosphorylated casein was degraded primarily to low molecular weight fragments, while those produced from

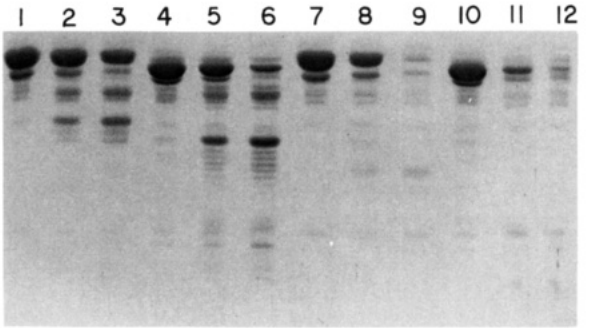


FIGURE 2: Degradation of casein and dephosphorylated casein by the multicatalytic proteinase complex. Each lane contained 20 μg of the protein substrate and 1.1 μg of the MPC. PAGE was carried out under denaturing and dissociating conditions. Lanes 1, 2, and 3, casein incubations at 0, 120, and 240 min, respectively. Lanes 4–6, dephosphorylated casein at the corresponding incubation times. Lanes 7, 8, and 9, casein in the presence of 0.04% SDS at 0, 60, and 120 min of incubation, respectively. Lanes 10–12, dephosphorylated casein at the corresponding incubation times.

dephosphorylated casein were at least initially less reactive, apparently because of initial accumulation of higher molecular weight fragments. This interpretation is supported by the pattern of degradation products visible in PAGE (Figure 2) in which the number of Coomassie Blue stainable products derived from dephosphorylated casein is markedly greater than that obtained from casein. Apparently, the products formed from casein were of lower molecular weight, and consequently more ninhydrin reactive, but less visible in PAGE. It is of interest that addition of SDS (0.04%) to incubation mixtures containing either casein or dephosphorylated casein greatly accelerated the proteolytic degradation of both proteins. This is clearly reflected both in the amount of ninhydrin-reactive material produced (Table VI) and also in the pattern of degradation visible in PAGE. Thus, SDS accelerated the degradation of casein after 60 min by almost 10-fold, and that of dephosphorylated casein by more than 100-fold. Indeed, PAGE indicated that both proteins were completely degraded after 120-min incubation (Figure 2). These results suggest that the proteolytic degradation in the presence of SDS is primarily catalyzed by the peptidylglutamyl-peptide hydrolyzing activity, since only this component was greatly activated by SDS in its activity toward synthetic substrates.

As indicated above, the degradation of lysozyme, serum albumin, and phosphorylase *b* proceeded at a much slower rate; nevertheless, SDS also markedly accelerated the degradation of these proteins. PAGE showed the formation of high molecular weight degradation products from lysozyme visible by

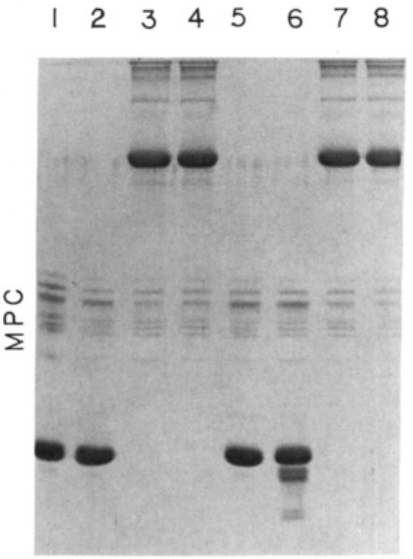


FIGURE 3: Degradation of lysozyme and human serum albumin by the multicatalytic proteinase complex. Lanes 1 and 2, lysozyme at 0 and 240 min of incubation, respectively. Lanes 3 and 4, albumin at the same incubation times. Lanes 5–8 are the corresponding experiments in the presence of 0.04% SDS. Other conditions are as in Figure 2. MPC = multicatalytic proteinase complex.

Coomassie Brilliant Blue staining (Figure 3); however, no such products could be detected in incubation mixtures containing serum albumin or phosphorylase *b* (not shown).

## Discussion

Bovine pituitaries represent an excellent source of the MPC because of the relative ease with which the enzyme can be isolated in a high degree of purity, and also because of the relative high activity of the final preparation toward both synthetic and protein substrates. The specific activities of the best preparations of the MPC toward Z-Gly-Gly-Leu-pNA were in the range of 16 μmol/h. This specific activity seems to be from 10 to 70 times higher than the specific activities reported for the MPC from other sources (Nojima et al., 1986; Wagner et al., 1986; Dahlman et al., 1985). While the activity of the pituitary enzyme is rather low when compared with the rate of degradation of synthetic substrates by such enzymes as trypsin and chymotrypsin, this activity should be evaluated with the understanding that any one of the proteolytic activities of the complex may be associated with only a single component with an average molecular mass of 26 kDa, and it may consequently constitute only a small fraction (about 1/30) of the total protein mass of the 700-kDa complex. Furthermore, the fact that the activity toward the synthetic substrates is not measured at saturating substrate concentrations, and that the reaction can be accelerated by a number of activators, points to a rather formidable proteolytic potential being associated with the complex. This conclusion is also supported by the rapid degradation of some proteins, such as casein and dephosphorylated casein (see Figure 2 and Table VI).

The basic properties of the pituitary enzyme are apparently representative of the MPC in other bovine tissues, although some subtle differences exist with respect to optimal temperature for cleavage and the activating effect of SDS (Ray & Harris, 1985, 1986, 1987). More distinct differences, however, seem to exist in the immunological properties and the electrophoretic banding pattern of MPC isolated from different species (Ray & Harris, 1987; Tanaka et al., 1988).

Electrophoresis of the bovine pituitary enzyme in high-resolution polyacrylamide gels under denaturing and dissociating conditions consistently leads to separation of eight major and three minor nonidentical subunits with molecular masses of 21–32 kDa. No higher molecular mass components have been found in the isolated enzyme. A similar composition has been reported for the complexes isolated from several other tissues (Ray & Harris, 1985; Hough et al., 1987), although the presence of as many as 15–20 subunits with isoelectric points in the range from pH 3 to 10 has been reported (Tanaka et al., 1988). The possibility, however, must be considered that some of the components may have arisen from limited proteolysis of the subunits by proteases of the complex, since such proteolytic degradation of the complex has been previously observed (Wilk & Orlowski, 1980). Posttranslational modification must also be considered. In several instances, the presence of higher molecular mass subunits ranging from 50 to 105 kDa has been reported (Schmid, 1984; Castaño et al., 1986; Zolfaghari et al., 1987; Nojima et al., 1986); however, these components have not been seen in highly purified preparations, and might therefore represent protein contaminants. No higher molecular mass components could be detected in the highly purified pituitary enzyme either by protein staining or by immunoreactivity with a polyclonal antibody raised in rabbits.

The pituitary enzyme is strongly inhibited by monovalent and divalent metal ions (Wilk & Orlowski, 1980, 1983). This property was also reported for the human lung enzyme (Zolfaghari et al., 1987); however, for the enzyme from other sources, no effect or even activation by monovalent cations was reported (Yamamoto et al., 1986).

The MPC from various sources including bovine pituitary was reported to be strongly inhibited by organic mercurials and thiol blocking agents (Wilk & Orlowski, 1980; Dahlman et al., 1985; Rivett, 1985; McGuire & DeMartino, 1986; Wagner et al., 1986; Zolfaghari et al., 1987) and to be quite resistant to inactivation by the generally used concentrations of DFP and phenylmethanesulfonyl fluoride. This finding led some authors to conclude that the enzymes of the complex belong to the class of thiol proteases (Dahlman et al., 1985; Rivett, 1985; Wagner et al., 1986). An expression of this classification was a proposal to name the enzyme "macropain" (McGuire & DeMartino, 1986), apparently to emphasize the belief that the enzyme might be related to papain, a thiol protease from papaya latex. Several authors, however, have noticed that higher concentrations of DFP (5–7 mM) and longer preincubation times inhibit the chymotrypsin-like activity of the complex (Wagner et al., 1986; Ishiura, 1986; Nojima et al., 1986); nevertheless, the remaining two proteolytic components were generally found to be resistant to inactivation by DFP, and therefore considered to be mechanistically different (Tanaka et al., 1986). The chymotrypsin-like activity of the pituitary enzyme is also inhibited by DFP at concentrations of 2–5 mM and like in enzymes from other sources its trypsin-like and peptidylglutamyl-peptide hydrolyzing activities are also resistant to DFP inhibition. All three activities, however, are rather rapidly inactivated by 3,4-dichloroisocoumarin (Harper et al., 1985), a general mechanism-based serine protease inhibitor, and also inhibited, albeit at a much slower rate, by other isocoumarin derivatives (Kam et al., 1988). These results indicate that all three enzymes of the complex apparently belong to the class of serine proteases. The finding that the $k_{obs}/I$ for 3,4-dichloroisocoumarin is much lower for the trypsin-like and peptidylglutamyl-peptide hydrolyzing activities than for the chymotrypsin-like activity is consistent with the finding that this last activity is the most sensitive to inhibition by DFP. The as-

signment of each of the proteolytic activities to defined components of the complex has not yet been accomplished. The lack, however, of inhibition of any of the proteolytic activities with a polyclonal antibody suggests that the active sites of the proteases of the complex are not freely accessible. The low inactivation rate with DFP and some isocoumarin derivatives in contrast to the rather rapid inactivation by others (such as 3,4-dichloroisocoumarin) may also result from a limited access of these molecules to the active site of the enzymes.

Preservation of the integrity of the MPC is apparently necessary for expression of its proteolytic activity. Attempts to dissociate the complex into active subunits have not been successful, suggesting that the complex represents a functional unit, although each of the components can be selectively activated. Thus, the chymotrypsin-like activity is activated by heparin, the peptidylglutamyl-peptide hydrolyzing activity is activated by SDS and fatty acids, and the trypsin-like activity is activated by DTT. Of particular interest was our previous finding that inhibition of the chymotrypsin-like activity toward Cbz-Gly-Gly-Leu-pNA, by the reversible transition-state analogue inhibitor Cbz-Gly-Gly-leucinal, led to a marked activation of the trypsin-like activity (Orlowski & Wilk, 1981; Wilk & Orlowski, 1983). In the present study, we report that irreversible inhibition of the chymotrypsin-like activity by low concentrations of 3,4-dichloroisocoumarin (Table III) also leads to a marked increase (more than 250%) in the trypsin-like activity. This finding further supports our previously expressed view that the MPC represents an interacting system, since these results imply that occupation of the active site of the chymotrypsin-like component accelerates proteolysis at the active site of the trypsin-like component.

The MPC rapidly degrades $\beta$-casein and dephosphorylated $\beta$-casein in a reaction greatly accelerated by SDS. The finding that both the rate of formation of ninhydrin-positive degradation products and also the nature of degradation fragments visible in PAGE are different for the two variants of casein indicates that the degree of phosphorylation greatly influences the rate and pattern of degradation of the proteins, apparently by changing the conformation of the proteins in such a way that the peptide backbone becomes more or less accessible to proteolytic degradation. The proteolytic degradation of oxidatively modified glutamine synthetase by the complex might be the result of a similar process (Rivett, 1985). The stimulation of degradation of caseins and also other proteins (Table VI) by SDS indicates that this reaction is mainly catalyzed by the peptidylglutamyl-peptide hydrolyzing activity, a component also shown to exhibit chymotrypsin-like activity.

The role of ATP in the degradation of proteins by the MPC remains a matter of controversy. In our experiments, ATP inhibited the activity of all three components of the complex toward small synthetic substrates (Table IV) and had no effect on the degradation of casein and dephosphorylated casein (data not shown). Several investigators, however, have reported stimulation by ATP of degradation of synthetic as well as protein substrates by preparations containing the MPC. Rechsteiner and his co-workers (Hough et al., 1987) reported on the purification from rabbit reticulocytes of two high molecular mass proteases. One of these having a molecular mass of about 1000 kDa was stimulated by ATP in its activity toward synthetic substrates and $^{125}$I-lysozyme–ubiquitin conjugates. The other, with a molecular weight of 700 000, was inhibited by ATP and had properties similar to if not identical with the enzyme isolated by us from bovine pituitary. The mechanism by which ATP stimulates activity is not clear; however, it seems that its effect is apparently due, at least in part, to stabilization of the protease against thermal inactivation (Hough et al., 1987). The same mechanism was also reported to explain the stimulatory effect of ATP on degradation of synthetic substrates by lysates of human erythroleukemia cells (Tsukahara et al., 1988), although it is not known whether these lysates contained both the high molecular weight proteases identified in rabbit reticulocytes and the 700-kDa MPC. In each case, however, it is rather unlikely that ATP was involved in the actual mechanism of cleavage of the peptide bond, since its effect was not dependent on the presence of $Mg^{2+}$ nor was this effect inhibited by chelating agents. This is consistent with the finding that the isolated MPC is either inhibited or not affected by ATP, as shown in our present study and also by others (Rivett, 1985; McGuire & DeMartino, 1986; Hough et al., 1987).

Finally, while the MPC isolated from various tissues and different species shows remarkable similarity with respect to molecular mass, subunit composition, and specificity toward synthetic substrates, significant differences seem to exist with respect to sensitivity to activators and inhibitors. Thus, for example, the enzyme isolated from rat liver cytosol is activated by polylysine and *N*-ethylmaleimide (Tanaka et al., 1986, 1988), the same compounds which markedly inhibit the pituitary enzyme (Wilk & Orlowski, 1980; Table IV in this report). Perhaps the most consistent similarity found for almost all of the enzymes so far studied is the activating effect of low concentrations of SDS and fatty acids on the peptidylglutamyl-peptide hydrolyzing activity. It remains to be established whether differences in sensitivity toward activators and inhibitors seen in enzymes from various sources reflect differences in functional aspects of the enzymes in various tissues.

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# Purification and Substrate Specificity of *Staphylococcus hyicus* Lipase†

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*Received February 6, 1989; Revised Manuscript Received June 21, 1989*

ABSTRACT: The *Staphylococcus hyicus* lipase gene has been cloned and expressed in *Staphylococcus carnosus*. From the latter organism the enzyme was secreted into the medium as a protein with an apparent molecular mass of 86 kDa. This protein was purified, and the amino-terminal sequence showed that the primary gene product was indeed cleaved at the proposed signal peptide cleavage site. The protein was purified from large-scale preparations after tryptic digestion. This limited proteolysis reduced the molecular mass to 46 kDa and increased the specific activity about 3-fold. Although the enzyme had a low specific activity in the absence of divalent cations, the activity increased about 40-fold in the prepsence of $Sr^{2+}$ or $Ca^{2+}$ ions. The purified lipase has a broad substrate specificity. The acyl chains were removed from the primary and secondary positions of natural neutral glycerides and from a variety of synthetic glyceride analogues. Thus triglycerides were fully hydrolyzed to free fatty acid and glycerol. The enzyme hydrolyzed naturally occurring phosphatidylcholines, their synthetic short-chain analogues, and lysophospholipids to free fatty acids and water-soluble products. The enzyme had a 2-fold higher activity on micelles of short-chain D-lecithins than on micelles composed of the L-isomers. Thus the enzyme from *S. hyicus* has lipase activity and also high phospholipase A and lysophospholipase activity.

Lipases (glycerol ester hydrolase, EC 3.1.1.3) and bacterial lipases in particular are widespread in nature. These enzymes hydrolyze triglycerides to diglycerides, monoglycerides, glycerol, and fatty acids. Interest in bacterial lipases has increased markedly in the last two decades. A large number of lipases have been screened for applications in medicine (digestive enzymes) or as food additives (flavor-modifying enzymes), industrial reagents (glyceride-hydrolyzing enzymes), and cleaners (detergent additives). Several lipases have been isolated and biochemically analyzed (Muruoka et al., 1982; Tyski et al., 1983; Rollof et al., 1987). These enzymes are characterized by a wide substrate specificity since they hydrolyze tri-, di-, and monoglycerides as well as poly(oxyethylene) sorbitan fatty acyl esters (Tweens).

The lipase gene of *Staphylococcus hyicus* has been cloned and expressed to high levels in *Staphylococcus carnosus* (Götz et al., 1985; Lechner et al., 1988). A preprotein with 641 amino acids was predicted from the DNA sequence with a molecular mass of 71.4 kDa, although the apparent molecular mass from SDS-PAGE gels was about 86 kDa. In *S. hyicus*, but not in *S. carnosus*, the 86-kDa protein was rapidly transformed into a 46-kDa form. In this paper we describe

† This work has been supported by the Dutch Organization for Advancement of Pure Science (NWO) and by the European Economical Community (BAP Contracts 0071-NL and 0196-D).
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0006-2960/89/0428-9278$01.50/0 