QUANTIFICATION OF Ca^{2+}-DEPENDENT PROTEASE ACTIVITIES BY HYDROPHOBIC AND ION-EXCHANGE CHROMATOGRAPHY

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ABSTRACT

Hydrophobic and ion-exchange chromatography were compared for yield of Ca^{2+}-dependent proteases and their inhibitor in studies designed to quantify Ca^{2+}-dependent proteases activity for comparative purposes. Ion-exchange (DEAE-Sephacel) proved superior to hydrophobic chromatography (Phenyl-Sepharose). Under the proper conditions, DEAE-Sephacel effectively separated low-calcium-requiring form of Ca^{2+}-dependent protease (CDP-I) and CDP inhibitor. Characterization of the assay system for components of the Ca^{2+}-dependent proteolytic system separated by ion-exchange chromatography indicated that proteolytic degradation of casein by Ca^{2+}-dependent proteases was linear with time for up to 60 min at 25°C and that it was linear up to .4 to .45 units of activity. Therefore, we recommend that, after identification of fractions containing Ca^{2+}-dependent protease (CDP-I or CDP-II), these fractions be pooled, and reassayed at a volume that yields values of less than .45 units of activity. Unlike CDP-I and CDP-II, CDP inhibitor lost its activity rapidly with frozen storage (frozen in liquid nitrogen, then stored at –70°C); therefore, inhibitor should be assayed in fresh (unfrozen) samples only.
(Key Words: Ca-Dependent Proteases, Inhibitors, Chromatography, Freezing.)

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Introduction

The Ca^{2+}-dependent protease initially was identified by Guroff (1964). This proteolytic system exists in all mammalian tissues thus far studied (Murachi et al., 1981; Goll et al., 1983). It was initially identified in skeletal muscle by Busch et al. (1972) and later purified by Dayton et al. (1976). Although the exact physiological functions of the calcium-dependent proteases are not known, in skeletal muscle they may play a key role in the degradation of myofibrillar proteins (Goll et al., 1983). This proteolytic system plays a key role in the tenderization process that occurs during postmortem storage of meat under refrigerated conditions (Koohmaraie et al., 1986, 1988a,b,c, 1989; Koohmaraie 1988, 1989).

Although numerous laboratories are conducting experiments examining the role of this proteolytic system in protein turnover in skeletal muscle and meat tenderization, the details of the procedures for extraction and determination of activities of the components of this proteolytic system have not been published. The objectives of this study were to determine which chromatographic methods are most appropriate for quantification of components of this proteolytic system for comparative studies and to determine optimum conditions for assaying their activities.

Materials and Methods

Preparation of Ca^{2+}-Dependent Proteases. Ca^{2+}-dependent proteases (CDP) were pre-

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pared from 100 g of longissimus muscle (from five Hereford × Angus crossbred animals) within 45 min of slaughter. Muscles, trimmed of visible fat and connective tissue, were homogenized in 3 volume of extraction solution that consisted of 50 m*M* Tris base, 10 m*M* EDTA and 10 m*M* 2-mercaptoethanol (MCE); pH was adjusted to 7.8 at 4°C with 6 *N* HCl. Minced tissue was homogenized with a Waring Blender[4], twice at low and twice at high speed, each for 30 s, with a 30-s cooling period interspersed between bursts. This homogenate was centrifuged at 28,000 × g_{max} for 120 min. The supernatant was filtered through four layers of cheesecloth and then glass wool (which had been washed with distilled and then deionized water) and pH was adjusted to 7.5. At this point the supernatant was divided into two equal volumes, one for quantification of CDP by hydrophobic and the other by ion-exchange chromatography.

Hydrophobic Chromatography. To the supernatant designated for hydrophobic chromatography, solid NaCl was added to final concentration of .5 *M*. The supernatant then was loaded at 1 ml/min onto a 1.6 × 40-cm column of Phenyl-Sepharose[5] that had been equilibrated with 50 m*M* Tris base, 1 m*M* EDTA, 10 m*M* MCE and .5 *M* NaCl with pH adjusted to 7.5 with 6 *N* HCl. Prior to loading, the supernatant was measured with a conductivity meter[6] to ensure that its conductivity was equal to that of the equilibrating buffer. After the sample was loaded, the column was washed with equilibrating buffer until the unadsorbed proteins were eluted completely (i.e., A_{278} of the outflow was ≤.1). The two CDP then were eluted with 50% (v/v) ethylene glycol in 50 m*M* Tris base, 1 m*M* EDTA and 10 m*M* MCE, with pH adjusted to 7.5 at 4°C with 6 *N* HCl.

Ion-Exchange Chromatography. To the supernatant designated for ion-exchange chromatography, ice-cold deionized water was added to reduce its conductivity to that of the equilibrated buffer for ion-exchange chromatography (50 m*M*Tris base, 1 m*M* EDTA and 10 m*M* MCE with pH adjusted to 7.5 at 4°C with 6 *N* HCl). The supernatant then was clarified by centrifugation at 105,000 × g_{max} for 60 min and loaded onto a 1.6-cm × 40-cm column of DEAE-Sephacel at 24 ml/h. After loading, the column was washed with equilibrating buffer (five column volumes) to remove unadsorbed proteins. The bound proteins then were eluted with a continuous gradient of NaCl from 0 to 400 m*M* (280 ml of each) in equilibrating buffer. Fractions were assayed for CDP-I, CDP-II and CDP inhibitor activities as described later.

Effect of Freezing on the Activities of Ca^{2+}-Dependent Proteases and Their Inhibitor. To study the effect of freezing on the activities of components of the Ca^{2+}-dependent proteolytic system, muscle samples were obtained from longissimus muscle of two Hereford × Brahman steers. Five hundred grams of lean muscle was obtained from each animal within 45 min of slaughter. Muscle samples, trimmed of fat and connective tissue, were cut into 2-cm^3 pieces. These pieces were mixed thoroughly and 50-g aliquots were obtained at random from the pool of 2-cm^3 pieces (2 determinations·animal^{-1}·freezing period^{-1}). These aliquots were either extracted immediately or frozen in liquid nitrogen and stored at –70°C for 2, 4 or 6 wk. At these specified times (0, 2, 4 and 6 wk), the samples were processed for preparation of Ca^{2+}-dependent proteases and their inhibitor by ion-exchange methods as described above.

Assay of Proteases and Their Inhibitor Activities. The activities of CDP-I and -II were determined using casein[7] as a substrate, as described by Dayton et al. (1976). This procedure determines the extent of proteolysis by measuring the amount of peptides released from casein by CDP, i.e., the increase in A_{278} in the soluble fraction after treatment with 2.5% trichloroacetic acid (TCA). The reaction mixture consisted of 100 m*M* Tris, 10 m*M* MCE and 5 mg/ml casein and 5 m*M* $CaCl_2$ with pH adjusted to 7.5 at 25°C with 1 *N* acetic acid. To determine Ca^{2+}-independent activity (i.e., materials that are soluble in 2.5% TCA independent of Ca^{2+}-concentration), the same reaction mixture, but containing 10 m*M* EDTA instead of 5 m*M* $CaCl_2$, was used. For specific details (i.e., incubation volume, incubation time) see figure legends. The reaction was initiated by adding CDP fractions and stopped by adding equal volumes of 5% TCA. After centrifugation at 2,000 × g_{max} for 30

[4]Dynamics Co. of America, New Hartford, CT.
[5]Pharmacia LKB, Piscataway, NJ.
[6]Type CDM 2 F, Radiometer America, Inc., Westlake, OH.
[7]U.S. Biochemical Co., Cleveland, OH.

min, the A_{278} of the supernatant fluid was determined. The A_{278} of the fractions in the presence of EDTA was subtracted from that in the presence of $CaCl_2$ to determine Ca^{2+}-dependent proteolytic activity. Total activity was calculated by multiplying Ca^{2+}-dependent proteolytic activity by the dilution factor. One unit of CDP activity is defined as the amount of enzyme that catalyzed an increase of 1.0 absorbance unit at 278 nm in 60 min at 25°C.

The inhibitor was determined by incubating appropriate amounts of inhibitor and CDP-II at 4°C for 1 min before adding 1.5 ml of reaction mixture containing $CaCl_2$ to start the reaction. The reaction was stopped with TCA and centrifuged as before. Three tubes were necessary to assay the inhibitor: a) protease alone (CDP-II); b) protease + inhibitor fraction in reaction mixture containing $CaCl_2$; and c) inhibitor alone in reaction mixture containing EDTA (to account for materials in the inhibitor fraction that are soluble in 2.5% TCA). Total inhibitor activity was calculated according to the following formula: total inhibition activity = a − (b − c) × dilution factor. One unit of inhibitory activity was defined as the amount of the inhibitor that inhibits one unit of CDP-II activity.

Results and Discussion

Comparison of Hydrophobic and Ion-Exchange Chromatography. The protease referred to as Ca^{2+}-dependent protease (CDP) in this manuscript has a variety of other names, including Ca^{2+}-activated factor (Busch et al., 1972; Olson et al., 1977; Koohmaraie et al., 1984, 1986), Ca_{2+}-dependent neutral proteinase (Vidalenc et al., 1983; Ducastaing et al., 1985), Ca_{2+}-activated protease (Suzuki et al., 1982), and Calpain (Murachi, 1985), among others (Goll et al., 1985). Two forms of the protease exist, one requiring micromolar concentration of calcium for activity (CDP-I) and the other requiring millimolar concentration of calcium (CDP-II). The third component of this proteolytic system is an endogenous inhibitor (CDP inhibitor or calpastatin) that inhibits the activity of both proteases (for review, see Koohmaraie, 1988).

Among nonlysosomal proteases, Ca^{2+}-dependent proteases have received a great deal of attention. They have been identified in all mammalian tissues thus far studied. Because of their involvement in the tenderization process and the role they may play in growth characteristics and meat quality of animals fed β-adrenergic agonists, the Ca^{2+}-dependent proteases have received a great deal of attention recently. In such studies, interest generally lies in determination of activities of the components of this proteolytic system for quantitative purposes (e.g., the levels of activity in controls vs animals fed β-adrenergic agonists). Although ion-exchange chromatography has been used routinely to separate the components of this proteolytic system, such a separation technique has been criticized, due to its inability to separate CDP-I from CDP inhibitor (see, e.g., Inomata et al., 1983; Yoshimura et al., 1983; Penny et al., 1985; Karlsson et al., 1985) and it has been argued that additional procedures are required to separate these proteins. Also, hydrophobic interaction chromatography (Phenyl-Sepharose) may be superior to ion-exchange chromatography for separation of CDP-I from CDP inhibitor when a crude preparation is used as the sample for chromatography (Inomata et al., 1983; Gopalakrishna and Barsky, 1985). In this manuscript we compared DEAE-Sephacel and Phenyl-Sepharose to examine which method was best for quantification of Ca^{2+}-dependent proteases using the same muscle extract (Figures 1 and 2). After loading and washing of the column to eliminate unbound proteins and CDP inhibitor, CDP-I and -II eluted in a single peak from Phenyl-Sepharose column. Figure 1 shows a representative chromatograph of Phenyl-Sepharose chromatography. A similar chromatograph was reported by Etherington et al. (1987) for beef muscle extract. Total CDP activity also was determined (Table 1). A representative chromatograph of DEAE-Sephacel chromatography is reported in Figure 2. Fractions containing activities of CDP-I, -II and inhibitor were pooled; total activities, assayed under optimal conditions, are reported in Table 1.

Results indicated that DEAE-Sephacel was by far superior to Phenyl-Sepharose (Table 1). Using five different muscle extracts, each from a different animal, the yield of CDP-I + CDP-II from Phenyl-Sepharose was only 53.3% that from DEAE-Sephacel chromatography (Table 1). In addition, DEAE-Sephacel chromatography effectively separated CDP-I from CDP inhibitor. In order to separate CDP-I from inhibitor, the total volume of the salt gradient must be at least five column volumes. If the

TABLE 1. COMPARISON OF CA^{2+}-DEPENDENT PROTEASE ACTIVITY FROM DEAE-SEPHACEL AND PHENYL-SEPHAROSE CHROMATOGRAPHY

	DEAE-Sephacel	Phenyl-Sepharose	% of DEAE-Sephacel removed by Phenyl-Sepharose
CDP-I[c]	53.84 ± 2.06[a]	ND[b]	
CDP-II[d]	53.84 ± 11.92	ND	
CDP-I + CDP-II	107.68 ± 13.50	56.82 ± 10.10	53.30 ± 11.64
CDP inhibitor[e]	172.64 ± 21.64	ND	

[a]Mean ± SD (data represent five separate preparations, each with muscle from a different animal).

[b]ND = not determined.

[c]Low Ca^{2+}-requiring Ca^{2+}-dependent protease. Total activity/50 g muscle (caseinolytic activity).

[d]High Ca^{2+}-requiring Ca^{2+}-dependent protease. Total activity/50 g muscle (caseinolytic activity).

[e]Inhibition of casein hydrolysis by CDP-II. Total activity/50 g muscle.

total volume of the gradient is less than five column volumes, CDP-I and CDP inhibitor may coelute partially. To ensure that there was no cross-contamination of the DEAE-Sephacel purified CDP-I and CDP inhibitor, fractions containing these proteins were pooled separately, dialyzed against elution buffer containing 5 m*M* EDTA, loaded on a DEAE-Sephacel column separately and eluted with a shallow gradient (0 to 200 m*M* NaCl). Results indicated that there was no detectable CDP inhibitor in DEAE-purified CDP-I and vice versa (data not shown). Based on these results, we recommend the use of ion-exchange chromatography for separating the components of the Ca^{2+}-dependent proteolytic system in studies designed to quantify these proteins for comparative purposes.

Optimum Conditions for Assaying CDP-I, CDP-II and CDP Inhibitor after Ion-Exchange Chromatography. To study the optimum condition for casein hydrolysis by Ca^{2+}-dependent protease, fractions containing CDP-II activity were pooled. Different amounts of protease activity (varied by volume of pooled fraction added to assay) and incubation times were studied. The rate of casein hydrolysis was linear (Figure 3) only up to .40 to .45 units of activity (A_{278} of the supernatant fluid, i.e., TCA-soluble peptides). Therefore, it is recommended that after the individual fractions containing CDP-I and CDP-II activity are identified, these fractions should be pooled separately and reassayed. A range of volumes should be assayed so that various amounts of protease activity are represented. Only a volume of pooled fraction producing activity of less than .4 to .45 absorbance units can be used legitimately to calculate total activity. Failure to observe this procedure could result in gross underestimation of CDP-I and CDP-II activities. Proteolytic activity of CDP-II, as a function of time of incubation at 25°C with casein as substrate, is reported in Figure 4. Caseinolytic activity of CDP-II was linear with time at 25°C up to 60 min. Although any time between 15 and 60 min is suitable for activity determination, we routinely use a 60-min incubation time to allow small quantities of CDP to express their activity.

The CDP inhibitor activities were determined according to the following procedures:

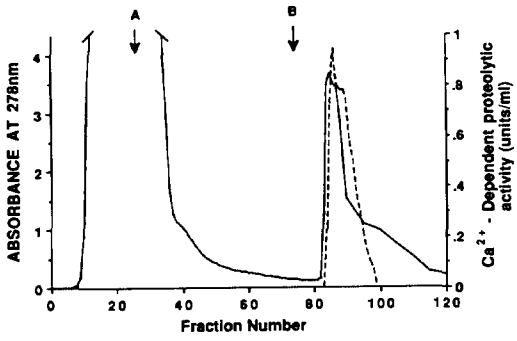

Figure 1. Chromatographic separation of low- and high-calcium-requiring forms of Ca^{2+}-dependent proteases using Phenyl-Sepharose. After loading the muscle extract, the column was washed (from A) with equilibrating buffer containing 500 m*M* until A_{278} of the outflow reached the baseline. At (B) the column was washed with equilibrating buffer containing 50% ethylene glycol to elute Ca^{2+}-dependent proteases. The Ca^{2+}-dependent proteases were eluted in a single peak. Column size: 1.6 × 40 cm; flow rate: 45 ml/h; fraction volume: 5.0 ml.

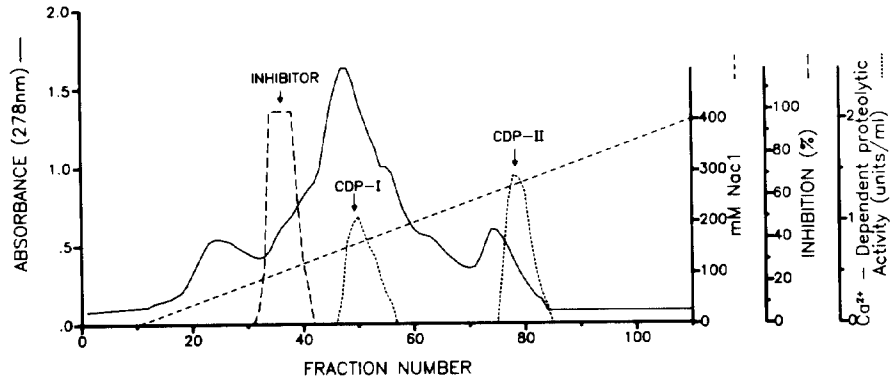

Figure 2. Chromatographic separation of low-and high-calcium-requiring forms of Ca^{2+}-dependent protease and their inhibitor using DEAE-Sephacel. 105,000 × g_{max} supernate of muscle extract was loaded onto DEAE-Sephacel column. After loading, the column was washed with equilibrating buffer until A_{278} of the outflow reached the baseline. The bound proteins were then eluted with a linear gradient of NaCl from 0 to 500 m*M*NaCl in equilibrating buffer. Column size: 1.6 × 40 cm; flow rate: 24 ml/h; fraction volume: 5.0 ml; gradient volume: 570 ml.

1) fractions 25 to 50 were assayed for inhibitor activity (Figure 2); 2) fractions containing inhibitor (i.e., 36 to 42) were pooled and reassayed at different volumes; 3) the volume that yielded 80% or less inhibitor activity was used to determine total inhibitor activities. Because the activity of CDP-I and CDP-II is linear only up to .4 to .45 units of activity, the quantity of Ca^{2+}-dependent protease (CDP-I or CDP-II; we normally use CDP-II) in the inhibitor assay must be adjusted so that in the absence of the inhibitor the assay yields an A_{278} of less than .4 to .45. Failure to follow these procedures could result in a gross underestimation of inhibitor activities.

Effect of Freezing on the Activities of Ca^{2+}-Dependent Proteases. In experiments in which numerous variables are measured, it is customary to freeze samples to alleviate excessive and unmanageable work loads, as we have done before with Ca^{2+}-dependent protease (Koohmaraie et al., 1987, 1988c). The effect of freezing (in liquid nitrogen, with storage at –70°C) on the activities of components of the Ca^{2+}-dependent protease system was examined (Table 2). Results indicate that whereas CDP-I and CDP-II were remarkably stable under these conditions, CDP inhibitor was unstable in the frozen state. The inhibitor had 66.6, 55.1 and 45.0% of its original activity after 2, 4 and 6 wk, respectively, at –70°C. Therefore, in studies designed to quantify CDP inhibitor activities, especially for comparative studies, freezing should be avoided, and separation and quantification of CDP inhibitor should be conducted with fresh muscle samples. The yield of CDP and inhibitor activities, particularly inhibitor, was considerably higher (Tables

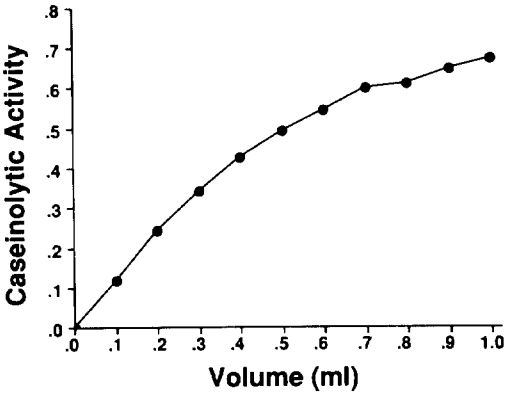

Figure 3. Proteolytic activity of high-calcium-requiring Ca^{2+}-dependent protease (CDP-II) with casein as substrate as a function of volume of the pooled DEAE-purified CDP-II. Total reaction volume was 2.5 ml and reaction stopped with 2.5 ml of 5% TCA. Incubation conditions: 60 min at 25°C. Experiment was done with four different preparations. Because of the disparity of activity in the different preparations, the result of a single preparation is reported graphically. The mean ± SEM of the correlation coefficient of the linear part of the curve for four preparations was .99 ± .01.

TABLE 2. EFFECT OF STORAGE AT -70°C ON THE ACTIVITIES OF CA^{2+}-DEPENDENT PROTEASES AND THEIR INHIBITOR[a]

Storage time, wk	CDP-I[b]	CDP-II[c]	CDP inhibitor[d]
0	44.6	48.3	211.9[f]
2	45.7	58.8	141.2[g]
4	45.9	58.2	116.8[h]
6	45.0	53.5	95.4[i]
RSD[e]	2.87	5.5	13.5

[a]Least square means.

[b]Low calcium-requiring Ca^{2+}-dependent protease. Total activity/50 g muscle (caseinolytic activity).

[c]High calcium-requiring Ca^{2+}-dependent protease. Total activity/50 g muscle (caseinolytic activity).

[d]Inhibitor of Ca^{2+}-dependent proteases. Total activity/50 g muscle (inhibition of casein hydrolysis by CDP-II).

[e]Residual standard deviation.

[f,g,h,i]Means within the same columns with different superscripts differ ($P<.05$).

1 and 2) than in some of our first publications (Koohmaraie et al., 1987, 1988c). The reason for these differences, in the case of inhibitor, we suspect, is primarily freezing of the samples prior to extraction and, in the case of CDP, assay under suboptimal conditions.

Implications

For quantification of the components of Ca^{2+}-dependent proteolytic system, ion-exchange (DEAE-Sephacel) is by far superior to hydrophobic (Phenyl-Sepharose) chromatography. However, hydrophobic chromatography is less time-consuming than ion-exchange chromatography and, therefore, might be a better separation technique for qualitative isolation of these proteins. Appropriate procedures should be followed during chromatography and determination of activities of these proteins to avoid underestimation of the activities of these proteins. Results indicate that these determinations, especially the quantification of CDP inhibitor, should use fresh muscle samples; freezing of muscle samples prior to chromatographic separation should be avoided.

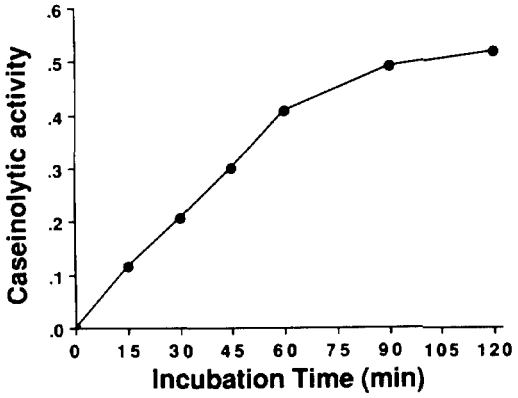

Figure 4. Proteolytic activity of high-calcium-requiring Ca^{2+}-dependent protease (CDP-II) with casein as a function of incubation time at 25°C. Source of protease: pooled DEAE-purified CDP-II. Experiment was done with four different preparations. Because of the disparity of activity in the different preparations, the result of a single preparation is reported graphically. The mean ± SEM of the correlation coefficient of the linear part of the curve for four preparations was .99 ± .01.

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