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STABILIZATION, PARTIAL PURIFICATION AND CHARACTERIZATION OF PEPTIDYL PEPTIDE HYDROLASES FROM GERMINATED BARLEY

W. C. BURGER, NEVILLE PRENTICE, MARY MOELLER and G. S. ROBBINS

Barley and Malt Laboratory, Crops Research Division, Agricultural Research Service, U.S. Department of Agriculture, Madison, Wisconsin, U.S.A.*

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Abstract—The requirements for the extraction and stabilization of several soluble peptidyl peptide hydrolases from germinated barley were determined. Two enzymes that are adsorbed by carboxymethyl cellulose (CMC) at pH 5·5 and a group of at least three enzmyes which are not adsorbed at this pH were separated by gel filtration. Some of the properties of these partially purified enzymes with respect to pH and thermal stability, the effect of ionic strength as well as various reagents were determined. All of the preparations hydrolyzed hemoglobin, casein and gliadin.

INTRODUCTION

BARLEY and barley malt were first shown in 1963 to contain at least five different peptidyl peptide hydrolases (endopeptidases) that attack gelatin.[1] Subsequent work with germinated barley in which hemoglobin and gelatin were used as substrates confirmed the presence of five such enzymes.[2,3] More recently, the use of extraction media of low pH was shown to be advantageous for obtaining relatively stable extracts of these hydrolases,[4,5] and the existence of two types of proteolytic enzymes in germinated barley was demonstrated: those inhibited by the sulhydryl reagent, *p*-chloromercuribenzoate, and lesser amounts which were inhibited by ethylenediamine tetraacetate.[4]

It is difficult to compare directly the results of these studies or to speak in terms of any particular peptidyl peptide hydrolase because the methods employed by individual investigators vary greatly and none of the individual enzymes has been separated and characterized. More definitive studies are necessary before the individual enzymes can be identified.

We have studied the soluble peptidyl peptide hydrolases of germinated barley with the object of separating and purifying them to a degree where extraneous proteins are less likely to interfere with the determination of some of the properties of these enzymes. The amount of activity extracted by an improved procedure is 200–300 per cent of that obtained in an earlier study.[3] The present paper describes the conditions which were found optimal for handling and storage of the enzymes, methods for their separation and purification, and the determination of some of the properties of individual enzymes.

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RESULTS AND DISCUSSION

Effect of Extraction Buffer pH

Preliminary experiments to determine if extraction of endopeptidase activity from germinated barley would be more efficient at relatively low pH were prompted by the observations that the optimum pH for hemoglobin digestion, 3·8–4·0, does not appear to be particularly deleterious to the enzymes involved, and that when excised barley aleurone tissue is incubated under essentially aseptic conditions in weakly buffered media, the pH of the incubation medium drops to 3·8 to 4·1 within 24 hr. The secretion of proteolytic activity under these conditions has been demonstrated.[6] Concurrent information[4] confirmed the advantages of using low pH.

To determine the effect of pH during extraction and dialysis, samples of finely ground, lyophilized, germinated barley were treated with buffers at pH 4, 4·5 and 5·0. The enzyme activity obtained over this narrow range of pH was essentially constant (Table 1). Less protein was extracted at pH 4·0 and 4·5, and a resultant increase in specific activity was observed as the pH was lowered. The use of still lower pH, e.g. 0·1 M ascorbate buffer, pH 3·5, did not yield as much activity and subsequent extracts were therefore made at pH 4.

TABLE 1. THE EFFECT OF pH UPON THE EXTRACTION AND STABILITY OF PROTEOLYTIC ACTIVITY

Buffer pH	Vol. (ml)	Activity (U/ml)	Total activity (U)	Protein (mg/ml)	Specific activity (U/mg protein)
4·0	89	6260	556,000	6·5	960
4·5	102	5430	554,000	6·5	840
5·0	97	5510	535,000	7·9	700

Purification of Peptidyl Peptide Hydrolases

Some of the peptidyl peptide hydrolases of germinated barley are relatively labile proteins. For this reason the use of gel filtration was found to be of some advantage over ion-exchange or adsorption column techniques for separating these proteins. Batch treatment on carboxymethyl cellulose (CMC), involving a relatively short adsorption period (20–30 min) at pH 5·5, was used as a preliminary means of dividing the proteins into adsorbed (CMC-a) and unadsorbed (CMC-u) fractions. The use of CMC columns with salt gradients, which has been of considerable value in peptide hydrolase studies,[7] was too severe a treatment for some of the more labile endopeptidases.

Because there are several barley peptidyl peptide hydrolases capable of hydrolyzing substrates such as hemoglobin and gelatin, a simple system for designating the individual enzymes was desirable. This is especially true during the early stages of study when details of enzymic or physical properties cannot always be determined. For the purposes of this discussion we have chosen to refer to these enzymes in terms of two important criteria for protein separation and purification, their acid-base properties and their molecular size. These properties are readily ascertained by determining the adsorptive effect of CMC at low ionic strength and the arbitrary pH of 5·5, and by the order of emergence of these enzymes from gel filtration media.

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The effluent diagram obtained when the CMC-a proteins were gel filtered on Sephadex G-100 is shown in Fig. 1. Endopeptidase assays with hemoglobin substrate indicated the presence of two active regions, one of low activity near the protein front, designated CMC-adsorbed I (CMC-aI), and a more active region associated with proteins of lower molecular weight, CMC adsorbed II (CMC-aII). Refiltration of the latter material on the same type

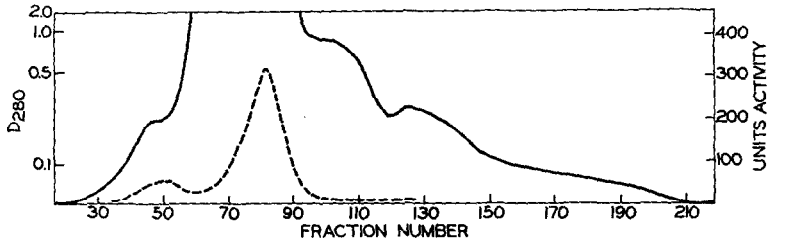

FIG. 1. EFFLUENT DIAGRAM OF CMC-a PROTEINS.
The solid line represents the optical density at 280 nm; the dashed line represents the proteolytic activity with hemoglobin substrate. 5 × 90 cm column, Sephadex G-100.

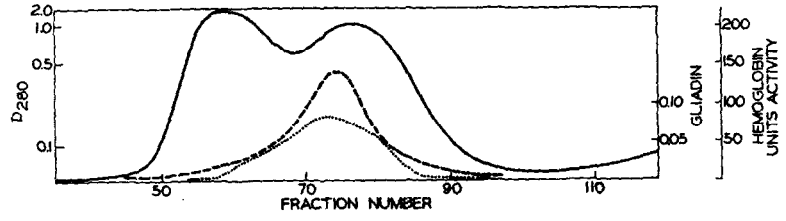

FIG. 2. EFFLUENT DIAGRAM OF SECOND PASSAGE OF CMC-aII THROUGH SEPHADEX G-100.
The dotted line represents the proteolytic activity measured with gliadin substrate; see Fig. 1 for other details.

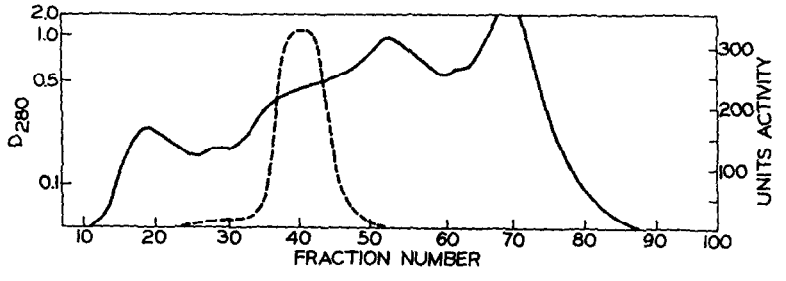

FIG. 3. GEL FILTRATION OF CMC-u PROTEINS.
See Fig. 1 for details. 5 × 87 cm column, Sephadex G-100.

of gel (Fig. 2) yielded a rather broad region of activity which represented approximately 50 per cent of that obtained from the first passage. The fractions under this peak were also tested for activity with gliadin substrate. The data in Fig. 2 show good correspondence between the activities exhibited with the two substrates with respect to the elution volume of the peaks.

Similar treatment of the more acidic CMC-u proteins produced effluent diagrams like that shown in Fig. 3. The endopeptidase activity was eluted in one peak, but the shape of the

Table 2. Purification of peptidyl peptide hydrolases

Fraction	Vol. (ml)	Total activity on		Total protein (mg)	Specific activities		Ratio specific activities (hemoglobin/casein)
		Hemoglobin (U)	Casein (U)		Hemoglobin (U/mg protein)	Casein (U/mg protein)	
Dialyzed extract	550	$2{\cdot}4\times10^{6}$	$5{\cdot}6\times10^{5}$	2200	1100	250	4·4
CMC-adsorbed	197	$1{\cdot}1\times10^{6}$	$5{\cdot}3\times10^{5}$	910	1200	580	2·1
CMC-aI (G-100)	25	$5{\cdot}4\times10^{4}$	$8{\cdot}6\times10^{4}$	98	550	860	0·6
CMC-aII (G-100)	29	$3{\cdot}1\times10^{5}$	$7{\cdot}9\times10^{4}$	188	1700	420	4·1
Re-run (G-75)	27	$9{\cdot}6\times10^{4}$	$4{\cdot}3\times10^{4}$	144	670	310	2·2
CMC-unadsorbed	59	$4{\cdot}5\times10^{5}$	$3{\cdot}4\times10^{4}$	94	4800	360	13·4
CMC-unadsorbed (G-75)	17·5	$5{\cdot}2\times10^{5}$	$1{\cdot}6\times10^{5}$	38	14,000	4200	3·3
Re-run (G-75)							
CMC-uI	7·5	$6{\cdot}3\times10^{4}$	$1{\cdot}5\times10^{4}$	6·1	10,000	2500	4·0
CMC-uII	4·4	$4{\cdot}8\times10^{4}$	$1{\cdot}2\times10^{4}$	3·7	13,000	3200	4·1

peak suggested the possible presence of more than one endopeptidase. When the active region was rechromatographed under conditions favoring better resolution, e.g. smaller column diameter, Sephadex G-75, and a micro flow cell, the enzyme activity pattern indicated that as many as three endopeptidases were present (Fig. 4). The results from assays of these fractions with casein substrate showed considerably less definition, but still provided confirmation of the presence of more than one enzymic component.

The distribution of proteolytic activity among the isolated enzyme preparations described above is summarized in Table 2. Data obtained with hemoglobin and casein substrates are included. The procedure yielded four preparations: CMC-aI; CMC-aII; and the unadsorbed enzymes which were partially resolved after passage through Sephadex G-75. For these experiments the unadsorbed enzymes were arbitrarily divided into two parts, the first, called CMC-uI, containing most of the first two endopeptidases to emerge from the column (Fig. 4), and the second part containing the major portion of the third endopeptidase peak, called CMC-uII. Recovery and degree of purification data for the various enzymes are not presented because of the difficulties involved when several enzymes of varying degrees of stability act on the same substrate. For this same reason, some of the specific activity values vary in an unexpected manner during the early stages of purification.

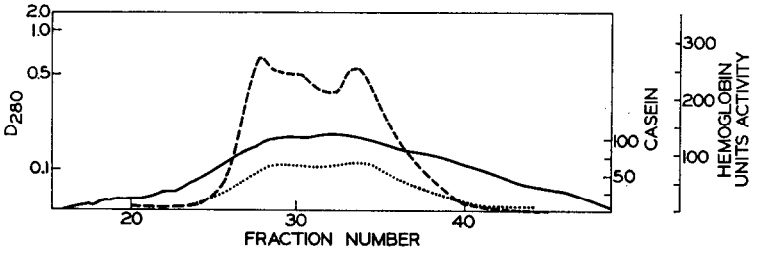

FIG. 4. SECOND GEL FILTRATION OF CMC-u ENDOPEPTIDASES.
The dotted line represents proteolytic activity with casein substrate; see Fig. 1 for other details. 2·5 × 81 cm column, Sephadex G-75.

From the ratios of activity on the two substrates shown in Table 2 it is apparent that CMC-aI and CMC-aII differ in their relative abilities to attack hemoglobin and casein. The more acidic enzymes, CMC-uI and CMC-uII, are very similar to one another in this respect.

The second passage of CMC-aII through gel resulted in sharp decreases in specific activity with hemoglobin substrate (Table 2). This is a reflection of the lability of this enzyme; subsequent tests (see below) revealed it to be the most labile of the four endopeptidase fractions.

There are some obvious differences in the results obtained with the two substrates. For example, gel filtration of the CMC-a fraction on Sephadex G-100 produced opposite effects upon the specific activity values of the two enzymes, CMC-aI and CMC-aII. A possible explanation for this would be the presence of a third enzyme which attacks casein preferentially and has little effect upon hemoglobin. Efforts to isolate such an enzyme have been initiated.

pH Stability of Peptidyl Peptide Hydrolases

The data of Table 3 show the effects of various buffers upon the CMC-u endopeptidases as a group, and the two CMC-u enzymes. The unadsorbed enzymes were optimally active

after treatment at pH 4 but showed a sharp drop in activity upon treatment at pH 3. Treatment at pH values above 4 does not appear to inactivate these enzymes so rapidly. The rather high value obtained with phosphate buffer at pH 6 is in sharp contrast to the values obtained at pH 7 and 8 with phosphate.

CMC-aI was relatively stable from pH 3 to pH 7 although its activity is somewhat lower at pH 5·5 in acetate. CMC-aII has a narrower pH range for stability and is optimally active near pH 4·5.

TABLE 3. STABILITY OF PEPTIDYL PEPTIDE HYDROLASES AT VARIOUS pH VALUES

Buffer	pH	Per cent activity remaining		
		CMC-u	CMC-aI	CMC-aII
HCl	1·2	2	—	—
Glycine	2·0	6	28	8
Glycine	3·0	48	82	64
Acetate	4·0	99	87	86
Acetate	4·5	—	78	107
Acetate	5·0	70	78	91
Acetate	5·5	—	54	56
Acetate	6·0	68	65	11
Phosphate	6·0	87	—	13
Phosphate	7·0	2	76	13
Phosphate	8·0	1	—	7
GFB, dialyzed	4·6	100	100	100

All buffers were $\mu=0{\cdot}11$ and contained 0·01 M cysteine. Gel filtration buffer (GFB), $\mu=0{\cdot}31$, was used as a control. Enzyme assays were accomplished with hemoglobin substrate. Treatment lasted 18 hr.

Effect of Ionic Strength upon Stability of Peptidyl Peptide Hydrolases

The CMC endopeptidases exhibit some increase in stability at higher ionic strengths (Table 4). CMC-aI shows very little effect in this respect with the exception of $\mu=0{\cdot}5$ where

TABLE 4. EFFECT OF IONIC STRENGTH UPON STABILITY OF PEPTIDYL PEPTIDE HYDROLASES

μ	Per cent activity remaining		
	CMC-u	CMC-aI	CMC-aII
0·5	88	58	123
0·15	88	89	84
0·05	63	82	74
0·015	54	82	74
0·005	56	80	63
GFB, dialyzed	100	100	100

Enzyme was dialyzed for 16 hr against acetate buffer, pH 4·5, containing 0·005 M cysteine, and of ionic strength as indicated. The dialyzed enzyme was removed from the tubing and stored under N_2 for 6 days before analysis with hemoglobin substrate. Control samples were treated similarly with gel filtration buffer (GFB), $\mu=0{\cdot}31$, pH 4·6, and contained the following activities: CMC-u, 1180 U per ml; CMC-aI, 2570 U per ml; and CMC-aII, 1520 U per ml.

activity dropped to approximately 60 per cent of the control sample. CMC-aII shows a tendency toward increased stability with increasing ionic strength and appears to be stimulated significantly at $\mu = 0{\cdot}5$.

Effect of Various Reagents upon Hydrolase Stability

Data on these effects for treatment periods of 18 hr and 7 days are presented; for many purposes the latter values would be more significant although some of the data obtained after 18 hr are of interest. The CMC-u enzymes (Table 5) showed transitory high stability in 0·5 M sucrose, 0·05 M cysteine and 0·01 M glutathione (GSH); however, aside from the gel filtration buffer, only 0·5 M sucrose remained relatively high during the longer treatment period. Ascorbate was particularly ineffective with these enzymes.

TABLE 5. STABILITY OF CMC-u PEPTIDYL PEPTIDE HYDROLASES IN PRESENCE OF VARIOUS REAGENTS

Treatment	pH	Per cent recovery	
		18 hr	7 days
GFB, undialyzed	4·60	100*	79
GFB, dialyzed	4·62	90	65
0·1 M acetate	4·03	76	56
ditto, plus 0·05 M sucrose	4·08	80	42
ditto, plus 0·5 M sucrose	4·02	119	72
ditto, plus 0·002 M cysteine	4·02	72	52
ditto, plus 0·01 M cysteine	4·11	74	55
ditto, plus 0·05 M cysteine	4·10	97	52
ditto, plus 0·01 M GSH	4·02	89	48
ditto, plus 0·01 M mercaptoethanol	4·02	76	50
ditto, plus 0·01 M thioglycolate	4·02	60	41
ditto, plus 0·01 M ascorbate	4·0	55	19

* 4760 U per ml.

TABLE 6. STABILITY OF CMC-aI PEPTIDYL PEPTIDE HYDROLASE IN PRESENCE OF VARIOUS REAGENTS

Treatment	pH	Per cent recovery	
		18 hr	7 days
GFB, undialyzed	4·70	100*	79
GFB, dialyzed	4·63	89	68
0·1 M acetate	4·48	89	65
ditto, plus 0·05 M sucrose	4·52	59	54
ditto, plus 0·5 M sucrose	4·53	86	56
ditto, plus 0·002 M cysteine	4·48	79	78
ditto, plus 0·01 M cysteine	4·44	101	64
ditto, plus 0·05 M cysteine	4·51	92	46
ditto, plus 0·01 M GSH	4·49	105	62
ditto, plus 0·01 M mercaptoethanol	4·50	97	63
ditto, plus 0·01 M thioglycolate	4·50	—	69
ditto, plus 0·01 M ascorbate	4·47	99	58

* 3770 U per ml.

CMC-aI (Table 6) showed little or no decrease in activity after 18 hr in the presence of the majority of the test solutions, but the gel filtration buffer and 0·002 M cysteine were superior after 7 days. In contrast to the CMC-u endopeptidases (Table 5), 0·5 M sucrose was of no particular value for maintaining CMC-aI. CMC-aII (Table 7) was the least stable and required 0·05 M cysteine for maximum stability after 7 days, although several reagents were quite effective during the 18 hr interval. In general, the conditions found most suitable for retention of enzyme activity in the unfrozen solutions were also found to be best for storing frozen preparations.

TABLE 7. STABILITY OF CMC-aII IN PRESENCE OF VARIOUS REAGENTS

Treatment	pH	Per cent recovery	
		18 hr	7 days
GFB, undialyzed	4·70	100*	73
GFB, dialyzed	4·68	95	81
0·1 M acetate	4·51	48	19
ditto, plus 0·05 M sucrose	4·57	79	19
ditto, plus 0·5 M sucrose	4·58	83	18
ditto, plus 0·002 M cysteine	4·50	59	50
ditto, plus 0·01 M cysteine	4·48	97	50
ditto, plus 0·05 M cysteine	4·55	107	84
ditto, plus 0·01 M GSH	4·50	66	42
ditto, plus 0·01 M mercaptoethanol	4·50	75	28
ditto, plus 0·01 M thioglycolate	4·50	56	27
ditto, plus 0·01 M ascorbate	4·48	77	28

* 3180 U per ml.

Thermal Stability

The effect of heating the three enzyme preparations at 35, 45 and 55° for 1 hr in gel filtration buffer is shown in Table 8. CMC-aI is the most stable of the three enzymes. CMC-aII is greatly reduced in activity after the treatment at 45°. The data obtained at 35° are of interest in so far as the temperature used for assaying these enzymes is concerned. The presence of substrate very likely has a stabilizing effect upon the enzyme, because no evidence of such thermal inactivation during assay has been observed.

TABLE 8. HEAT STABILITY OF PEPTIDYL PEPTIDE HYDROLASES

Enzyme	Per cent activity at		
	35°	45°	55°
CMC-u	78	79	3
CMC-aI	92	83	82
CMC-aII	83	19	3

EXPERIMENTAL

Materials

Lyophilized germinated barley. *Hordeum vulgare*, variety Trophy, 1965 crop from Madison, Wisconsin, was treated as previously described.[8]

Determination of Peptidyl Peptide Hydrolase Activity

Hemoglobin substrate. 2 ml of 1% (w/v) hemoglobin substrate powder in 0·1 M acetate buffer, pH 3·8, 0·1 ml of 0·31 M thioglycollic acid, 0·9 ml of water and 0·1 ml of enzyme solution were incubated at 35° for 60 min. 3 ml of 10% (w/v) TCA were added to stop the reaction and the mixture was held for 30 min at 35° and filtered on a 9 cm circle of Whatman No. 40 filter paper. 1 ml of the filtrate was analyzed for N according to the procedure of Johnson[9] except that the digestion catalyst was omitted. Controls to which the enzyme was added after the TCA had been added were used for all samples. A unit of enzyme is that amount which produces 1 μg of nitrogen under the above conditions. Appropriate changes in the volume of the enzyme solution used for incubation or the volume of the filtrate used for analysis were made when necessary. As with most methods of this type, a linear relationship between the amount of enzyme used and the amount of product produced was not obtained when relatively crude enzyme preparations were employed.[10] As the individual enzymes were purified, however, the shape of the enzyme concentration curve approached linearity.

Casein substrate. An adaptation of the method of Witt and Tousignant[11] was used. The reaction mixture contained 5 ml substrate solution, 1·9 ml of the buffer, pH 6·0, and 0·1 ml enzyme solution. This was incubated at 35° for 30 min and the reaction was stopped with the addition of 3 ml 30% (w/v) TCA solution. The mixture was held for an additional 30 min at 35° and filtered through Whatman No. 40 filter paper. Filtrates from reaction mixtures and appropriate controls (enzyme added after TCA solution) were read against a water blank at 280 nm. A unit of enzyme is defined as that amount of enzyme which releases 1 μg of tyrosine. Amounts of enzyme solution varying from 50 to 200 μl were used and the volume of buffer used was adjusted accordingly.

Gliadin substrate. The method of Jacobsen and Varner[6] was employed.

Purification of Peptidyl Peptide Hydrolases

Preliminary experiments to compare the efficacy of extraction at low pH were performed with 50-g quantities of finely ground germinated barley which were extracted for 1 hr with 125 ml of 0·1 M acetate buffers, pH 4·0, 4·5 and 5·0, and containing 0·01 M ascorbic acid. After centrifugation at 12,000 × *g* for 30 min the supernatant liquid was dialyzed against 2 × 1300 ml of the respective extraction buffers for 16 hr. The dialyzed samples were recentrifuged and used for enzymic and protein analyses (Table 1).

The following purification procedure includes several improvements which were found to produce increased yields of the enzymes in question, or which resulted in increased stability of the products. All operations were done in the cold and enzyme solutions and gel filtration buffers were held under high purity nitrogen whenever possible.

Extraction of germinated barley. 200 g of freshly ground lyophilized, germinated barley were extracted with 0·1 M acetate, pH 4·0, that was 0·05 M with respect to cysteine. The pH of the mixture was adjusted to 4·0 with 6 N HCl. The extraction mixture was held in a covered container that was flushed with N_2 and held in an ice bath for 1 hr with occasional stirring. Centrifugation was done in 250-ml covered bottles for 30 min at 14,000 × *g*.

Dialysis. The resulting supernatant (approximately 375 ml) was adjusted to pH 4·5 with 5 N NaOH and 125-ml portions were dialyzed against 3 × 1300 ml of 0·005 M acetate buffer, pH 5·5, that was 0·005 M with respect to cysteine for a period of 16 hr on a revolving wheel (1 rev/min).

Batch treatment with CMC. The retentate was centrifuged at 14,000 × *g* for 15 min and the clarified supernatant (*ca.* 500 ml) was treated with 350 g damp CMC 11 that was previously equilibrated with 0·005 M acetate buffer, pH 5·5. The CMC-u proteins were removed from the CMC on a suction funnel and the CMC washed with three 400-ml portions of the dialysis buffer. The pH of this fraction was adjusted to 4·5. The CMC-a proteins were eluted without delay from the CMC with three 450-ml portions of 0·5 M acetate buffer, pH 5·5, which was 0·005 M with respect to cysteine. This fraction was also adjusted to pH 4·5. Both fractions were concentrated by ultrafiltration to 100–150 ml.

Gel filtration. The CMC-a and CMC-u protein fractions were gel filtered on a 5 × 90 cm column of Sephadex G-100 by upward flow at 45 ml per hr with a gel filtration buffer (GFB) which consisted of 0·1 M acetate buffer, 0·2 M NaCl, 0·01 cysteine and 0·004 M EDTA at pH 4·7. Effluent was monitored at 280 nm. Fractions

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[11] P. WITT and E. TOUSIGNANT, *Cer. Chem.* **44**, 403 (1967).

(12–15 ml) containing the respective peptidyl peptide hydrolases were pooled, concentrated, and rechromatographed on a 2·5 × 90 cm column of the same gel at 20 ml per hr with upward flow. Effluent from the smaller column was monitored at 280 nm with a Vanguard Analyzer equipped with micro flow cells of 40 μl capacity for increased resolution. When appropriate, a second passage through the smaller column was employed. The use of Sephadex G-75 was found to be advantageous for separating the CMC-u fraction. Repeated gel filtration, however, resulted in severe losses in activity despite the precautions taken to stabilize the enzymes.

Treatment of Enzymes with Solutions of Various pH, Ionic Strength and Activators

Samples of enzyme (0·5–1·0 ml) were dialyzed in 8/32 Visking dialysis tubing against 125 ml of the solution containing the test substance on a revolving wheel (1 rev/min) at 2°. The dialysis bag plus contents were weighed prior to and after treatment to measure any volume change, the contents were assayed, and a correction made for any volume change. Results are expressed in terms of the undialyzed sample held in the gel filtration buffer at 2°.

Treatment of Enzymes at Various Temperatures

1 ml portions of enzyme solutions was held under N_2 at 35, 45 or 55° for 1 hr in stoppered tubes. Enzyme activities were compared with the corresponding unheated enzyme.

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