A Carboxypeptidase from Germinated Barley and Its Action on Casein

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A carboxypeptidase has been separated from the known proteases of germinated barley by column chromatography on Sephadex G-100 and carboxymethyl cellulose. This enzyme released the C-terminal amino acids from various CBZ-dipeptides, hippuryl-L-phenylalanine, and α- and β-casein and also hydrolyzed hippuryl-DL-β-phenyllactate. It showed no endopeptidase or dipeptidase activity. The enzyme was most stable in succinate buffer in the pH range 3 to 6. Cysteine had a slightly stabilizing effect on enzyme activity, potassium bromate produced partial inhibition, and *p*-chloromercuribenzoate produced inhibition varying from 22 to 100%, depending on the substrate used. Cu^{2+}, Ni^{2+}, and Zn^{2+} showed a small inhibitory effect and Fe^{3+} was strongly inhibitory.

In studying the endopeptidases of green malt, an enzyme which hydrolyzed casein but which had negligible activity on hemoglobin was found (Burger *et al.*, 1969b). The enzyme was separated from the known endopeptidases and peptide hydrolases of green malt. Its action on casein and on various synthetic peptide substrates indicates it is a carboxypeptidase. This paper describes the separation of the enzyme, some of its properties, and a preliminary study of its substrate specificity.

MATERIALS AND METHODS

Reagents used in this study were of analytical grade or the highest grade commercially available, and solutions were prepared with glass-distilled water. All operations were done at 0 to 10° C; enzyme solutions and buffers containing reducing agents were kept under high-purity nitrogen.

Lyophilized, germinated barley, *Hordeum vulgare* L., variety Trophy, 1965 crop from Madison, Wis., was prepared as previously described by Burger *et al.* (1968). One hundred fifty grams of freshly ground green malt was extracted for 1 hr at pH 4.0 with 300 ml of 0.1 *M* sodium succinate containing 0.05 *M* cysteine. After centrifugation for 30 min at 14,000 G, the supernatant (approximately 230 ml) was titrated to pH 4.5 with about 2 *N* NaOH. The solution was divided into two portions which were each dialyzed for 16 hr on a revolving wheel (1 rpm) against 4 × 1300 ml of 0.01 *M* sodium succinate buffer, pH 4.5, containing 0.005 *M* cysteine, and centrifuged for 15 min. The combined supernatant was concentrated to about 75 ml by ultrafiltration. This was done by placing the sample in dialysis tubing (No. 8, Visking Co., Chicago, Ill.) at atmospheric pressure. The pressure outside the casing was then reduced to about 25 to 50 mm of Hg. No enzyme activity was detected in the ultrafiltrates.

This solution was applied and eluted at about 60 ml per hr by upward flow from a 5 × 85 cm column of Sephadex G-100 equilibrated with the above dialysis buffer. The effluent was monitored automatically at 280 nm. Fractions were collected at 20 min intervals and assayed. The fractions which had activity on casein were pooled, concentrated by ultrafiltration, and centrifuged at 14,000 G.

Carboxymethyl cellulose (CMC) (CM 52, Reeve Angel Co., Clifton, N.J.) was thoroughly washed with 0.5 *N* NaOH, distilled water, 0.05 *N* HCl, and again with water. The washed CMC was equilibrated with 0.01 *M* sodium succinate, pH 4.5, containing 0.005 *M* cysteine, and was used to form a column 2.5 × 85 cm. The enzyme solution was applied to the column by upward flow at the rate of 24 to 28 ml per hr and eluted with a gradient formed by drawing 350 ml of 0.5 *M* succinate, pH 4.5, into 350 ml of 0.01 *M* succinate, pH 4.5, as the latter was pumped on the column. After the gradient, additional 0.5 *M* succinate, pH 4.5, was pumped on the column. All buffers used contained 0.005 *M* cysteine. The effluent was monitored and fractions collected as before. Fractions were assayed for activity on both casein and hemoglobin.

Activity on casein (Hammersten quality casein, Nutritional Biochemicals Corp., Cleveland, Ohio) was measured by an adaptation of the method of Witt and Tousignant (1967). Enzyme solution (50 to 400 μl which had been diluted to 2.0 ml with cysteine-EDTA-phosphate buffer, pH 6.0) was incubated for 30 min at 35° C and then filtered. The filtrates from the reaction mixtures and controls (enzyme added after TCA) were read at 280 nm. A unit (U) of enzyme activity was defined, similar to that suggested by Witt and Tousignant (1967), as the μg of tyrosine released in 30 min.

The above assay was designed for use with endopeptidases, and, as such, gives a linear relationship between enzyme concentration and activity. However, when the assay procedure is used with a carboxypeptidase, it is sensitive only to the highly-absorbing tryptophan released from the C-terminal end of α_{s1}-casein (see Discussion). The casein concentration used is not large enough to make the decrease in terminal tryptophan negligible; it cannot be increased because of the limited solubility of casein at pH 6 and below. Therefore activity falls off at high enzyme concentrations and no more than 150 to 200 units of enzyme were used per assay. The reaction rate also decreases with time as shown in Figure 1.

A modified assay with casein was used for some experiments. It was identical to the standard procedure above except that EDTA and cysteine were omitted from the buffer solution and that the casein solution was titrated to pH 6.0 with 1 *N* HCl instead of citric acid. Activity measured was comparable to that obtained by the basic assay.

Endopeptidase activity on hemoglobin substrate was assayed as previously described by Burger *et al.* (1969b); activity on gliadin substrate was assayed by the method of Jacobsen and Varner (1967). Assays for the hydrolysis of L-leucyl-β-naphthylamide, *N*-benzoyl-DL-arginine-*p*-nitroanilide, *N*-benzoyl-L-arginine ethyl ester, and α-naphthyl acetate were done by the procedures of Burger *et al.* (1968, 1969a). Protein was determined by the method of Lowry *et al.* (1951); crystalline bovine plasma albumin was used for calibration.

To determine the effects of different buffers, reagents, and pH on the stability of the enzyme, 0.6 ml samples were dialyzed

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Table I. Purification of Barley Carboxypeptidase

Fraction	Vol. (ml)	Activity on Hemoglobin (U/0.1 ml)	Activity on Casein (U/0.1 ml)	Protein (mg/ml)	Total Activity on Hemoglobin (U)	Total Activity on Casein (U)	Total Protein (mg)	Specific Activity on Hemoglobin (U/mg)	Specific Activity on Casein (U/mg)	U on Csn / U on Hbn
Dialyzed extract	320	530	230	4.6	1.69×10^6	7.5×10^5	1470	1150	510	0.44
Concentrated ext.	120	790	410	7.8	9.4×10^5	4.9×10^5	940	2010	530	0.52
From G-100	61	490	330	5.6	3.0×10^5	2.0×10^5	340	880	600	0.68
From CMC	46	[a]	160	1.1	[a]	7.4×10^4	50	...	1450	...

[a] Negligible.

for 16 hr at 0 to 4° C against 2 × 175 ml of various solutions on a revolving wheel (1 rpm). Activities on casein were compared after allowances for small changes in volume were made. The effect of metal ions was studied by incubating for 30 min 0.2 ml samples of enzyme solution with 2 ml of 0.05 *M* phosphate buffer containing 2×10^{-4} to 2×10^{-2} *M* metal chloride. After incubation, casein solution was added and the assay carried out with the modified assay procedure.

For analyses of reaction filtrates for phosphate, carbohydrate, and ninhydrin-positive substances (Table IV), two concentrations of enzyme were assayed in quadruplicate using the modified assay with casein in succinate buffer. The absorbance of the filtrates was measured at 280 nm. One ml samples of the TCA filtrates were analyzed for nitrogen according to the method of Johnson (1941), except that the digestion catalyst was omitted. The TCA filtrates were tested for ninhydrin-positive materials by the method of Witt and Tousignant (1967), except that the filtrates were diluted five-fold instead of twenty-fold before assay. One ml of the filtrate was analyzed for carbohydrate by the method of Dubois *et al.* (1956) and for total phosphorus by the method of Fiske and SubbaRow as described by Leloir and Cardini (1957), except that hydrogen peroxide was used instead of nitric acid for decolorizing.

Ion-exchange chromatography was performed on an apparatus constructed according to that of Spackman *et al.* (1958) using Aminex H resin in a scheme similar to that of Benson and Patterson (1965). The TCA filtrates analyzed were from a modified assay on casein in which the enzyme-substrate mixture was incubated for 45 min.

Carboxypeptidase activity with hippuryl-L-phenylalanine substrate at 5×10^{-4} *M* was assayed by the method of Folk and Schirmer (1963). The method of McClure *et al.* (1964) was used with 10^{-3} *M* hippuryl-DL-β-phenyllactate as substrate. The rates of hydrolysis of various CBZ-dipeptide substrates were measured by the procedure of Visuri *et al.* (1969) with enzyme which had been heated in a boiling water bath for 5 min used for the controls. In experiments with *p*-chloromercuribenzoate (PCMB) as inhibitor, 0.2 ml enzyme was incubated for 60 min at 30° C with 0.75 ml of appropriately diluted PCMB in buffer. The reaction was then started by the addition of 0.25 ml of 8×10^{-3} *M* substrate.

The hydrolysis of dipeptide, CBZ-dipeptide, and hippuryl-L-phenylalanine substrates at pH 5.2 in 0.05 *M* sodium succinate buffer was determined by thin-layer chromatography of the reaction mixtures as previously described by Burger *et al.* (1968). The enzyme solution contained 82 U per 0.1 ml and had been dialyzed against 3 × 1300 ml of 0.05 *M* succinate buffer, pH 5.2.

RESULTS

The purification procedure and the results of a typical preparation are summarized in Table I. Recoveries are not included because much of the hydrolysis of casein by the extract is associated with the endopeptidases reported previously by Burger *et al.* (1969b). For the same reason the apparent degree of purification appears relatively low. The product obtained from CMC chromatography had negligible activity on hemoglobin at pH 3.8; this is evident from Figure 2, which shows the elution pattern obtained in the 0.3 to 0.5 *M* region of the buffer gradient. The first two peaks which are active on casein and hemoglobin contain endopeptidases; the third peak active on casein but with negligible activity on hemoglobin contains the enzyme. This peak was also found to have no measurable activity on gliadin.

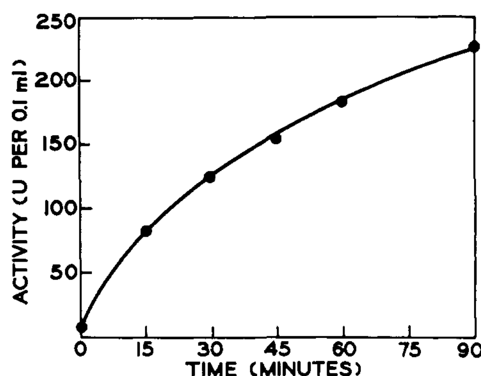

Figure 1. Relation between reaction time and carboxypeptidase activity on casein. Enzyme solution contained 125 U per 0.1 ml

Succinate buffers were superior to acetate buffers under otherwise identical conditions; approximately one half as much activity was recovered when acetate buffers were used in the procedure outlined in Figure 2, and the specific activity of the final product was about one third of that obtained with succinate buffers.

Dialysis of the enzyme for 16 hr against various buffers including succinate decreased activity on casein, sometimes as much as 40%. Similar or even greater losses were found on concentration by ultrafiltration through dialysis tubing. From the position of the enzyme during elution from Sephadex G-100, it seemed unlikely that the enzyme molecule was small enough to pass through the dialysis tubing; in fact, an assay of a filtrate from ultrafiltration failed to reveal the missing activity. It is not known if the observed losses in activity were caused by adsorption on the dialysis tubing or by denaturation.

Freezing also caused large losses in activity. A preparation of the enzyme containing 246 U per 0.1 ml which had been frozen immediately after elution from CMC contained only 56% of its original activity after 4 weeks, 23% after 6 weeks, and no activity after 6 months. Enzyme solutions stored unfrozen at 0 to 2° C lost their activity less rapidly than frozen solutions and were relatively active for several weeks.

Table II. Effect of pH and Buffer Ions on Enzyme Stability[a]

Buffer, 0.05*M*	pH	% Activity Recovered
Glycine (Cl)	2.0	0
Glycine (Cl)	3.0	95
Succinate (Na)	3.5	123
Succinate (Na)	4.0	119
Succinate (Na)	4.5	100
Succinate (Na)	5.0	95
Succinate (Na)	5.95	104
Phosphate (K, Na)	6.0	121
Phosphate (K, Na)	7.0	75
Tris (Cl)	8.0	11
Tris (Cl)	9.0	28

[a] Enzyme control contained 148 U per 0.1 ml. Samples were dialyzed 16 hr, and all buffers contained 0.005 *M* cysteine.

Table III. Effect of Various Reagents on Enzyme Stability

Reagent	% Recovery
0.01 *M* cysteine[a]	114
0.01 *M* cysteine[b]	117
0.05 *M* cysteine[b]	113
0.01 *M* β-mercaptoethanol[a]	84
0.01 *M* thioglycollic acid[a]	91
0.001 *M* EDTA[a]	79
0.005 *M* EDTA[a]	81
0.002 *M* $KBrO_3$[b]	83
0.01 *M* $KBrO_3$[b]	70
0.05 *M* $KBrO_3$[b]	73
0.25 *M* sucrose[a]	86

[a] Control contained 152 U per 0.1 ml. Samples were dialyzed 16 hr in 0.05*M* succinate, pH 4.5, and assayed with the standard assay. [b] Control contained 55 U per 0.1 ml. Samples were dialyzed 16 hr in 0.02*M* succinate, pH 4.5, and assayed with the modified assay.

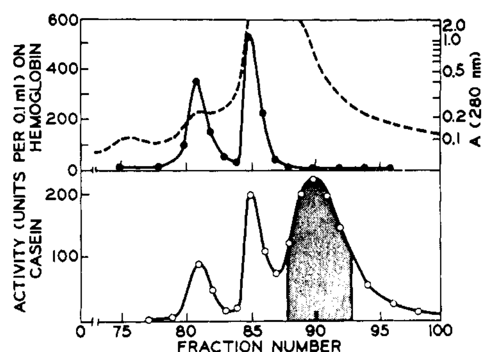

Figure 2. Elution of carboxypeptidase from CMC column

The portion shown is eluted by 0.3 to 0.5 *M* succinate buffer, pH 4.5. The shaded area indicates the carboxypeptidase. Absorbance, ---------; Activity on casein, O—O; Activity on hemoglobin ●—●

The enzyme was relatively stable at high temperatures and its activity on casein was not affected after 1 hr at 45° C in succinate buffer, pH 4.5, containing 0.005 *M* cysteine. After 1 hr at 55° C, activity decreased 59%. After 18 hr at 25 or 35° C, activity decreased about 20%, compared to a sample which had been stored in an ice bath.

The effect of pH and buffer ions on the stability of the enzyme after dialysis for 16 hr at 4° C is shown in Table II. The values are calculated on the basis of the sample dialyzed at pH 4.5 being valued at 100%. Maximum stability was observed in the range pH 3 to 6. Above pH 6, activity declined gradually with increasing pH; however, some activity remained after treatment at pH 9.

The effects of various reagents upon activity on casein are shown in Table III. These values are also based upon controls that were dialyzed in 0.02 or 0.05 *M* succinate buffer, pH 4.5, being placed at 100%. Cysteine stabilized the enzyme slightly; however, other reducing reagents, EDTA, or sucrose reduced stability somewhat. The stabilizing effect of cysteine was also studied using the modified assay with the same results. Potassium bromate appeared to have only a minor inhibitory effect, even at concentrations as high as 0.05 *M*.

Various metal cations at 2×10^{-4} and 2×10^{-3} *M* produced no stimulation of activity on casein. Ca^{2+}, Mg^{2+}, and Mn^{2+} had little or no effect, Cu^{2+}, Ni^{2+}, and Zn^{2+} produced a low degree of inhibition, and Fe^{3+} was strongly inhibitory at the above concentrations.

The apparent specificity of the enzyme for casein relative to hemoglobin and gliadin prompted an examination of the hydrolysis products obtained with casein. The TCA-soluble nitrogen released did not compare with that expected from endopeptidase activity as shown in Table IV. No measurable carbohydrate or phosphate was released. The approximate agreement of the μmoles of ninhydrin-positive material, calculated as leucine, with the μmoles of total nitrogen released (Table IV) suggested that the very limited action of the enzyme on casein was releasing a relatively high proportion of aromatic amino acids or cysteine.

Table IV. Absorption, Total Nitrogen, and Ninhydrin-Positive Nitrogen in the TCA Filtrates from Casein Reaction Mixtures[a]

Enzyme (μl)	Absorption 280 nm (ΔA)	Total Nitrogen (Δμmole N)	Ninhydrin-Positive N (Δμmole Leu.)
200	0.081 ± 0.014	0.6 ± 0.2	0.8 ± 0.4
400	0.155 ± 0.018	1.5 ± 0.7	2.1 ± 0.2

[a] Assays were carried out in succinate buffer. All quantities are expressed in terms of 10 ml of filtrate and are differences between reaction and control. The enzyme sample contained 58 U per 0.1 ml.

Table V. Amino Acids Released from Casein by Barley Carboxypeptidase

Product	μmoles per liter filtrate	E mol. (280 nm)	Calculated A (280 nm)
Tryptophan	51.6	5550	0.286
Leucine	24.3	...	...
Valine	29.5	...	...
Isoleucine	32.2	...	...
Tyrosine	2.6	1340	0.003
Phenylalanine	3.2	92	0.000
Peptides	none	...	...

To determine which amino acids were being released by the enzyme, TCA filtrates from a casein reaction mixture and its control were analyzed for amino acids by ion-exchange chromatography. This showed that only six amino acids were produced, two of them in much less quantity than the other four (Table V). The lack of any peptides indicates that the enzyme does not, in fact, show any endopeptidase activity, but instead attacks terminal amino acids of the casein molecule. The difference in absorbance at 280 nm between the reaction and control filtrates analyzed was 0.32. The calculated absorption of tyrosine, 0.003, and tryptophan, 0.286, essentially account for the absorption of the filtrates at this wavelength.

An examination of the action of the enzyme on substrates commonly used with carboxypeptidase A revealed that esterase activity, as measured using hippuryl-DL-phenyllactic acid,

was significantly greater than peptidase activity, as measured using hippuryl-L-phenylalanine. Other dipeptide substrates with blocked amino groups revealed varying degrees of activity, as shown in Table VI. Of the nine CBZ-dipeptides examined, CBZ-Ile-Phe was the most susceptible, CBZ-Gly-Leu and CBZ-Glu-Tyr were somewhat less easily hydrolyzed, while CBZ-Ile-Ala, CBZ-Leu-Gly, and CBZ-Gly-Tyr were considerably less reactive. Examination by thin-layer chromatography of the reaction mixtures from 16 hr incubations of enzyme with each of these substrates showed that only the original C-terminal amino acid was released. Similar tests for hydrolysis of Tyr-Gly, Ala-Ser, Ala-Val, Ala-Phe, Ala-Gly-Gly, Gly-Trp, Gly-Tyr, Leu-Gly-Gly, Leu-Gly, Gly-Leu, and Gly-Gly were all negative. The enzyme showed no measurable hydrolysis of L-leucyl-β-naphthylamide, N-benzoyl-DL-arginine-p-nitroanilide, N-benzoyl-L-arginine ethyl ester, or α-naphthyl acetate, which are substrates for known peptide hydrolases from green malt (Burger *et al.*, 1968, 1969a).

The relative rates of hydrolysis of the CBZ-dipeptides by the barley carboxypeptidase described by Visuri *et al.* (1969) have been included in Table VI. Some significant differences in the specificities of the two carboxypeptidases are evident. If the activity of each enzyme with CBZ-Ile-Phe is used as a reference, the activity of our enzyme on CBZ-Glu-Tyr is only 60% of that with the reference compound, whereas the enzyme of Visuri *et al.* hydrolyzed it nearly ten times as fast as the reference compound. Similarly, CBZ-Gly-Ala is not hydrolyzed by our enzyme, but the enzyme of Visuri *et al.* hydrolyzed it at nearly four times the rate of the reference compound. Other differences in the specificities of the two enzymes were also observed with CBZ-Gly-Leu, CBZ-Ile-Ala, and CBZ-Leu-Gly.

The effect of PCMB upon enzyme activity was determined with the three CBZ-dipeptides which were most susceptible to hydrolysis. At an inhibitor concentration of 10^{-3} M, the degree of inhibition was found to vary with the particular substrate employed. The following percentage activity values, relative to the untreated control, were obtained: CBZ-Glu-Tyr, 78%; CBZ-Ile-Phe, 44%; and CBZ-Gly-Leu, 9%.

Table VI. Relative Rates of Hydrolysis of CBZ-Dipeptides by Barley Carboxypeptidase

Substrate	Relative Rates of Hydrolysis: Present Enzyme	Relative Rates of Hydrolysis: Carboxypeptidase of Visuri *et al.*[a]
CBZ-Ile-Phe	100	100
CBZ-Gly-Leu	68	225
CBZ-Glu-Tyr	60	950
CBZ-Ile-Ala	20	100
CBZ-Leu-Gly	20	100
CBZ-Gly-Tyr	12	
CBZ-Gly-Trp	0	
CBZ-Gly-Pro	0	0
CBZ-Gly-Ala	0	375
Hippuryl-Phe[b]	23	

[a] Calculated from Visuri *et al.* (1969). [b] 10^{-3} M instead of 2×10^{-3} M.

DISCUSSION

The release of the C-terminal amino acids from hippuryl-L-phenylalanine and several CBZ-dipeptides, and the lack of any action on simple dipeptides, N-acyl derivatives of arginine, or L-leucyl-β-naphthylamide and α-naphthyl acetate show that the enzyme which has been separated from germinated barley is a carboxypeptidase.

Visuri *et al.* (1969) reported that germinated barley apparently contains two or possibly several carboxypeptidase-like enzymes on the basis of substrate specificity tests. They found that unfractionated extract hydrolyzed CBZ-Ile-Phe, CBZ-Gly-Leu, CBZ-Glu-Tyr, and CBZ-Ile-Ala significantly faster than their purified enzyme. These are the substrates which were hydrolyzed most rapidly by the carboxypeptidase described in this paper. The differences in relative activities with several CBZ-dipeptides for the two carboxypeptidases suggest they are different enzymes.

The enzyme of Visuri *et al.* was completely inactivated after 2 hr at pH 8.0 (30° C); the present enzyme retained 28% activity after 16 hr at pH 9.0 (4° C) (Table II). The carboxypeptidase described by Visuri *et al.* was also stable when stored frozen and apparently withstood dialysis with only moderate losses in activity. Our enzyme was not stable in the frozen state and exhibited rather large losses (30 to 40%) upon dialysis at pH 4.5 for 16 hr at 4° C. These differences indicate that germinated malt contains at least two carboxypeptidases which differ in their substrate specificities and in stability upon purification.

A comparison of the amino acids released from casein by this barley carboxypeptidase with the C-terminal amino acids of α-, β-, and κ-caseins shows a close correlation. Kalan *et al.* (1964) showed that α_{s1}-casein, the major protein fraction in casein, has an N-terminal arginine and a C-terminal sequence of Leu-Trp-OH. Kalan *et al.* (1965) showed that β-casein, second only in concentration to α-casein in cow's milk, has an N-terminal arginine and a C-terminal sequence of Ile-Ile-Val-OH. Dumas (1968) reported a C-terminal sequence of Ser-Val-Thr-Ala-Val-OH for κ-casein, and a recent paper by Jolles *et al.* (1969) suggests an N-terminal glutamine. Dumas (1968) has shown that carboxypeptidase A released the C-terminal amino acids from individual caseins equally well in a mixture of these caseins. When carboxypeptidase A acted on whole casein, amino acids were produced from the α_{S1}-, β-, and κ- fractions; tyrosine and a small amount of phenylalanine were also produced. Tyrosine presumably originated from the α_{S2}- and α_{S3}-caseins which have a C-terminal sequence of Leu-Tyr-OH according to Dumas (1968), and phenylalanine from the small amounts of *para*-κ-casein present in whole casein.

The products of hydrolysis of casein by the barley enzyme are very similar to those produced by carboxypeptidase A. Thus the first two amino acid products in Table V correspond to the products of hydrolysis of the C-terminal end of α_{S1}-casein. The next two amino acids in the table are consistent with the hydrolysis of the C-terminal end of β-casein. The small amounts of tyrosine and phenylalanine correspond to that found by Dumas (1968). Some valine is probably produced by the hydrolysis of κ-casein; however, the greater concentration of β-casein than κ-casein, the fairly large yield of isoleucine (from β-casein), and the absence of a measurable amount of alanine (from κ-casein) suggest that a major part of the valine was produced from the hydrolysis of β-casein. This picture is also consistent with the idea that the assay using casein as substrate is sensitive only to the highly-absorbing tryptophan released from the C-terminal end of α_{S1}-casein. It is evident from the absence of arginine and glutamine in the TCA filtrates (Table V) that the enzyme does not attack the N-terminal end of any of these casein fractions. Thus it would appear that this enzyme is a carboxypeptidase with action on casein similar to that of carboxypeptidase A.

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