THE EFFECT OF CALCIUM ON CHYMOTRYPSINS *a* AND B*

by

FENG CHI WU AND M. LASKOWSKI

Department of Biochemistry, Marquette University School of Medicine, Milwaukee, Wis. (U.S.A.)

The enhancing effect of calcium on the activity of trypsin has recently been studied by several workers[1–5]. Only GREEN *et al.*[4] extended their studies to α-chymotrypsin. They found that the activity of α-chymotrypsin catalyzing the hydrolysis of 0.01 *M* acetyl-L-tyrosine ethyl ester was increased to 150% of the initial level by the presence of 0.01 *M* calcium ion in the system. During our studies of the action of naturally occurring trypsin inhibitors on chymotrypsins *a* and B[6], it was noticed that the activities of both chymotrypsins were influenced by the presence of calcium ions in the medium, but to different degrees. It was therefore decided to study this phenomenon in a somewhat more detailed manner, in order to establish optimum conditions for the assay of each chymotrypsin. The results of these studies are the subject of the present paper.

METHODS

α-Chymotrypsin was prepared by the method of KUNITZ AND NORTHROP[7], and chymotrypsin B was prepared by the methods previously described[8,9]. Both enzymes were recrystallized 4 times.

The activity was tested by three different assay methods.

1. Spectrophotometric method of KUNITZ[10], in which casein prepared according to DUNN[11] was used as substrate, and the incubation time at 37° was reduced to 10 minutes. In order to investigate the effect of calcium, 0.1 *M* phosphate buffer, originally recommended by KUNITZ was replaced by 0.1 *M* borate buffer.

2. Method of ANSON[12], as modified by GREEN AND WORK[13]. Urea denatured, commercial hemoglobin (Difco) was used as a substrate in 0.1 *M* borate buffer; the activity was followed by reading the optical density at 280 mμ, after precipitating the non-digested hemoglobin with trichloroacetic acid and centrifuging the sample in a Sorvall SS-1 centrifuge for 10 minutes.

3. Method of PARKS AND PLAUT[14], in which L-phenylalanine ethyl ester hydrochloride (PhEE), purchased from Mann Research Laboratories, Inc., served as substrate. A blank containing no enzyme was included in each set of experiments, since a small, but significant hydrolysis of substrate was observed under our experimental conditions.

RESULTS AND DISCUSSION

In previous publications from this laboratory[15,8,16], the activity of chymotrypsin B (on the basis of weight) was reported to be consistently lower than the activity of α-chymotrypsin. Since both enzymes have the same molecular weight[9], it also meant a lower specific activity of chymotrypsin B. The only exception was found recently[6]

* Aided by a research grant from the National Institute of Arthritis and Metabolic Diseases National Institutes of Health, United States Public Health Service.

References p. 115.

when the activities of both chymotrypsins were compared using synthetic substrate (PhEE) in presence of 0.003 M $CaCl_2$. In this system chymotrypsin B was significantly more active.

The experiments performed on urea-denatured hemoglobin in the presence of variable amounts of calcium in the media indicated that the relative activity of chymotrypsin B might be increased beyond that of α-chymotrypsin. Fig. 1 illustrates the results of such an experiment performed in the absence and in the presence of 0.1 M $CaCl_2$. In the absence of calcium, chymotrypsin B showed about 85% of the activity of α-chymotrypsin. The inclusion of 0.1 M $CaCl_2$ in the medium increased activities of both enzymes, but the activity of chymotrypsin B was increased more than that of α-chymotrypsin. As a result, in the presence of 0.1 M $CaCl_2$ the activity of chymotrypsin B was somewhat higher than that of α-chymotrypsin.

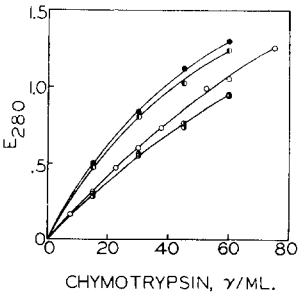

Fig. 1. Effect of calcium on activities of chymotrypsins as measured by the hemoglobin method[12,13]. ○ – α-chymotrypsin without Ca, ◑ – chymotrypsin B without Ca, ◐ – α-chymotrypsin in 0.1 M $CaCl_2$, ● – chymotrypsin B in 0.1 M $CaCl_2$. Final concentrations in the incubation mixture 1.2% hemoglobin, 0.05 M borate buffer pH 7.5, enzyme γ/ml as indicated, total volume 2 ml. Incubation time 5 minutes at 37°.

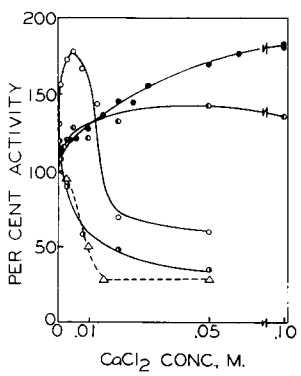

Fig. 2. Effect of calcium concentration on the activity of chymotrypsins. Activity without Ca is taken as 100%. Manometric assays with PhEE according to[14] at 30°, pH 6.5. Final concentrations in the reaction mixture are 0.025 M PhEE, 0.042 M $NaHCO_3$, 0.003 M borate buffer, calcium concentration as indicated. Assays with casein according to[10] at 37°, 0.05 M borate, pH 8.0, incubation time 10 minutes. Each point represents an average of 3 determinations at 3 different concentrations of enzyme. ○ – α-chymotrypsin with casein, ◑ – chymotrypsin B with casein, ◐ – α-chymotrypsin with PhEE, ● chymotrypsin B with PhEE. The dotted line represents casein remaining in solution at Ca concentrations indicated on abscissa. Numerical values on the ordinate for this curve refer to % of soluble casein instead of % activity.

Still more striking results were obtained with PhEE as substrate. In this case even in the absence of calcium, the activity of chymotrypsin B was about 30% higher than that of α-chymotrypsin. The difference was strongly accentuated by increasing the calcium concentration in the medium, and with 0.1 M $CaCl_2$, the activity of chymotrypsin B was about *twice* that of α-chymotrypsin (Figs. 5 and 2).

The results of the experiments in which the chymotrypsin activity was plotted versus the calcium concentration of the medium are summarized in Fig. 2. The curves obtained with hemoglobin are omitted for the purpose of clarity, since they were almost identical with the curves obtained with PhEE, with the exception that they were about 5 to 10% lower. The similarity of results between hemoglobin and PhEE

protease and esterase activities of chymotrypsin. The introduction of calcium increased the sensitivity of both methods to a similar extent, and it seems highly probable that it will similarly increase the sensitivity of any other method, provided the substrate remains soluble at the optimal concentration of calcium, and no complicating side-reaction occurs.

The data obtained with PhEE as substrate at variable calcium concentrations and 3 different levels of chymotrypsin B are plotted in Fig. 3. Assuming that calcium reacts reversibly with chymotrypsin to form calcium-chymotrypsin complex: $n\,[Ca^{++}] + [Ch] \rightleftarrows [Ca_n\,Ch]$, and that at any calcium concentration the activity observed is directly proportional to $[Ch] + \alpha\,[Ca_n\,Ch]$, where α is the ratio of activities of chymotrypsin-calcium complex to chymotrypsin; the relation between the activity and calcium concentration could then be expressed by the following equation:

$$\frac{\text{activity observed}}{\text{activity } [Ca^{++}] = 0} = 1 + (\alpha - 1)\,\frac{K\,[Ca^{++}]^n}{1 + K\,[Ca^{++}]^n}$$

Of all perimeters tested, the best fitting theoretical curve (solid line) was that drawn with the following perimeters: $n = 1$ (uniunimolecular reaction), $K_{assoc.} = 50$, $\alpha = 2$. In spite of the fact that the deviation of experimental points from the theoretical curve is larger than might be desired, the data are consistent with the assumption of a uniunimolecular reaction.

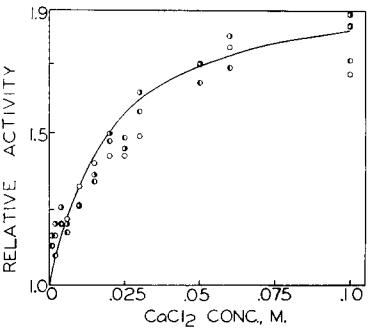

Fig. 3. Effect of calcium on the activity of chymotrypsin B as measured manometrically with PhEE, pH 6.5, 30°. Relative activity is plotted on the ordinate taking the activity without Ca as 1.0. Final concentration of chymotrypsin B, ○ – 18 γ/ml, ◐ – 30 γ/ml, ◑ – 42 γ/ml. Solid line represents the theoretical curve for a uniunimolecular reaction (see text) with values $\alpha = 2.0$, $K_{assoc.} = 50$.

Whereas essentially the same results were obtained with hemoglobin and PhEE as substrate, the results obtained with casein differed significantly (Fig. 2). In this case optimal activities were found at a much lower range of calcium concentration than in the previous two cases. Furthermore, the optimum calcium concentration was different for each enzyme, and the effect was reversed (α-chymotrypsin activity was enhanced much more than chymotrypsin B).

The sharp decline of activity represented by the descending arm of the curve is apparently caused by the low solubility of our preparation of casein in the presence of calcium. The increasing amounts of $CaCl_2$ were added to several tubes containing the standard solution of casein. The tubes were centrifuged in a Sorvall SS-1 centrifuge for 30 minutes at full speed, and the amount of casein remaining in solution was read in a Beckman spectrophotometer at 280 mμ. The results showed that the increase in calcium concentration from 0.005 M to 0.015 M decreased the soluble casein from 95 to 28% of the original. Further increase in calcium concentration had but little effect on the solubility of the remaining casein (Fig. 2).

No satisfactory explanation can be offered at present for the unusually pronounced effect of low calcium concentrations on digestion of casein by α-chymotrypsin. The abnormal behavior of different caseins as proteolytic substrates has been recently investigated[17], and the occurrence of side-reactions has been suggested. If one postulates that casein once clotted becomes more accessible to further hydrolysis by chymotrypsins, one might expect that the chymotrypsin with a higher clotting activity will show a higher activity, as measured by the formation of small fragments soluble in

The clotting activities of chymotrypsins were measured in a Beckman spectrophotometer at 37°. One per cent solution of casein in 0.1 *M* acetate buffer, pH 5.6, and an equal volume of the solution of enzyme were mixed, and the optical density was read at 30 second intervals at 800 mμ. The course of the reaction (density plotted versus time) followed an S-shaped curve, with the inflection point in the neighborhood of $E_{800}^{1\text{ cm}} = 1.0$. This point was therefore chosen, and the time required to reach the density of 1.0 was recorded. Fig. 4 shows that the reciprocal of this time is almost directly proportional to the enzyme concentration. The results, presented in Fig. 4, also show that casein clotting ability of α-chymotrypsin is over twice that of chymotrypsin B. It was not possible to investigate the effect of increased Ca concentrations in this system since 0.0005 *M* calcium produced precipitate without the added enzyme.

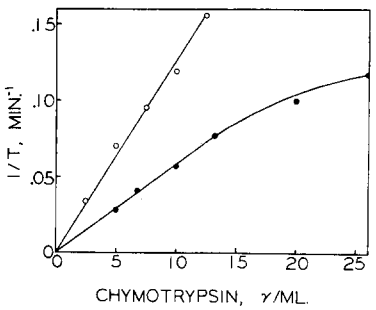

Fig. 4. Comparison of casein-clotting ability of chymotrypsins. Beckman spectrophotometer at 37°, corex cells 1 cm wide. 1.5 ml 1% casein in 0.1 *M* acetate buffer, pH 5.6 mixed with 1.5 ml enzyme solution. The optical density at 800 mμ is read against the blank containing no enzyme at frequent intervals. The time required to reach the optical density of 1.0 is recorded and the reciprocal is plotted on the ordinate. ○ – α-chymotrypsin, ● – chymotrypsin B.

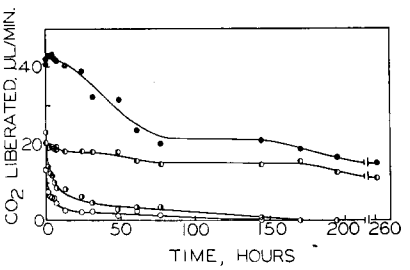

Fig. 5. Effect of calcium on the stability of chymotrypsins at 37°. Solution of α-chymotrypsin 200 γ/ml, chymotrypsin B 600 γ/ml, in 0.05 *M* borate buffer, pH 7.9. At indicated times samples were withdrawn and assayed manometrically at pH 6.5, according to[14], other assay conditions as described in Fig. 2. All samples were assayed at an enzyme concentration of 20 γ/ml. Samples containing no Ca were assayed without Ca; α-chymotrypsin protected by Ca was assayed at a final $CaCl_2$ concentration of 0.05 *M*, and chymotrypsin B at 0.1 *M*. ○ – α-chymotrypsin without Ca, ◑ – chymotrypsin B without Ca, ◐ – α-chymotrypsin with 0.5 *M* $CaCl_2$, ● – chymotrypsin B with 0.5 *M* $CaCl_2$.

Finally, the stabilities of dilute solutions of chymotrypsins at 37°, pH 7.9, in the absence, and in the presence of 0.5 *M* $CaCl_2$ were compared, Fig. 5. The results show that calcium exerted a favorable effect on stability of both chymotrypsins. Of the two, α-chymotrypsin was more stable in the presence of calcium. In the absence of calcium chymtrypsin B was somewhat more stable, retaining 36% activity after 13 hours, 30% after 20 hours of exposure, whereas the respective figures for α chymotrypsin were 22 and 18%.

Preliminary experiments in which partially autolyzed chymotrypsin was subjected to paper electrophoresis did not reveal the appearance of a second protein spot. When samples from the partially autolyzed enzyme were taken at different time intervals and were chromatographed on paper (Whatman No. 3) in *n*-butanol: formic acid: water = 75:15:10; numerous ninhydrin positive, small fragments were detected as early as 1 hour after the exposure of enzyme solution to 37°. As the autolysis proceeded, the intensity of the protein spot decreased, and the concentration of the small frag-

References p. 115.

ments increased. Addition of calcium to the medium delayed the appearance of hydrolysis products.

ACKNOWLEDGEMENTS

Our thanks are due to Dr. M. PRIVAT DE GARILHE for performance of paper chromatography, to Mr. H. A. HAESSLER for paper electrophoresis, and to Dr. M. LASKOWSKI, Jr., for stimulating suggestions and discussions.

SUMMARY

The observation of GREEN *et al.*[4] that the presence of calcium in the reaction mixture enhanced the activity of α-chymotrypsin has been confirmed. Calcium exerted even stronger effect on the activity of chymotrypsin B. Both chymotrypsins were stabilized by the presence of calcium in the medium. In order to increase the sensitivity and reliability of the assay methods using substrates like hemoglobin, L-phenylalanine ethyl ester (PhEE), and probably many others, the solubility of which is not influenced by calcium, the inclusion of 0.05 *M* $CaCl_2$ is recommended for α-chymotrypsin, and 0.1 *M* $CaCl_2$ for chymotrypsin B. When casein is used as substrate, 0.0005 *M* $CaCl_2$ is recommended for chymotrypsin B, and 0.005 *M* for α-chymotrypsin.

Contrary to previous results from this laboratory indicating that chymotrypsin B was consistently less active than α-chymotrypsin, in a system containing 0.1 *M* $CaCl_2$ and PhEE as substrate, at 30°, pH 6.5, the activity of chymotrypsin B was approximately twice that of α-chymotrypsin.

RÉSUMÉ

L'observation de GREEN *et al.*[4], selon laquelle la présence de calcium dans le milieu réactionnel augmente l'activité de l'α-chymotrypsine a été confirmée. Le calcium a un effet encore plus prononcé sur l'activité de la chymotrypsine B. Les deux chymotrypsines sont stabilisées par la présence de calcium dans le milieu. En vue d'accroître la sensibilité et la fidélité des méthodes de dosage utilisant des substrats comme l'hémoglobine, l'ester éthylique de la L-phénylalanine (PhEE) et probablement beaucoup d'autres, dont la solubilité n'est pas influencée par la présence de calcium, l'introduction de 0.05 *M* $CaCl_2$ est recommandée dans le cas de l'α-chymotrypsine et 0.1 *M* $CaCl_2$ dans le cas de la chymotrypsine B. Lorsque la caséine est utilisée comme substrat, 0.0005 *M* $CaCl_2$ est recommandé dans le cas de la chymotrypsine B et 0.005 *M* dans le cas de l'α-chymotrypsine.

Contrairement à des résultats provenant de ce laboratoire et indiquant que la chymotrypsine B était régulièrement moins active que l'α-chymotrypsine dans un système contenant 0.1 *M* $CaCl_2$ et PhEE comme substrat, à 30° C, pH 6.5, l'activité de la chymotrypsine B était, en fait, double de celle de l'α-chymotrypsine.

ZUSAMMENFASSUNG

Die Beobachtung von GREEN *et al.*[4], dass die Gegenwart von Kalzium die Aktivität des α-Chymotrypsins erhöht, wurde bestätigt. Auf Chymotrypsin B übte Kalzium einen sogar noch grösseren Einfluss aus. Beide Chymotrypsine wurden in Gegenwart von Kalzium stabilisiert. Um die Empfindlichkeit und Zuverlässigkeit der Bestimmungsmethoden zu erhöhen, in denen Substanzen wie Haemoglobin, L-Phenylalaninäthylester, und vielleicht viele andere, benutzt werden deren Löslichkeit von Kalzium nicht beeinflusst wird, wird die Beifügung von 0.05 *M* $CaCl_2$ zu α-Chymotrypsin, und von 0.1 *M* $CaCl_2$ zu Chymotrypsin B empfohlen. Bei Kasein ist die empfohlene Konzentration 0.0005 *M* $CaCl_2$ für Chymotrypsin B und 0.005 *M* für α-Chymotrypsin.

Im Gegensatz zu früher aus diesem Laboratorium mitgeteilten Beobachtungen konnte jetzt festgestellt werden, dass die Wirkung des Chymotrypsins B auf L-Phenylalaninäthylester in Gegenwart von 0.1 *M* $CaCl_2$ bei 30° und pH 6.5 ungefähr zweimal so gross ist wie die des α-Chymotrypsins.

References p. 115

REFERENCES

1 L. GORINI, *Biochim. Biophys. Acta*, 7 (1951) 318.
2 M. BIER AND F. F. NORD, *Arch. Biochem. Biophys.*, 33 (1951) 320.
3 W. G. CREWTHER, *Australian J. Biol. Sci.*, 6 (1953) 597.
4 N. M. GREEN, J. A. GLADNER, L. W. CUNNINGHAM JR. AND H. NEURATH, *J. Am. Chem. Soc.*, 74 (1952) 2122.
5 N. M. GREEN AND H. NEURATH, *J. Biol. Chem.*, 204 (1953) 379.
6 F. C. WU AND M. LASKOWSKI, *J. Biol. Chem.*, 213 (1955) 609.
7 M. KUNITZ AND J. H. NORTHROP, *J. Gen. Physiol.*, 19 (1936) 991.
8 K. D. BROWN, R. E. SHUPE AND M. LASKOWSKI, *J. Biol. Chem.*, 173 (1948) 99.
9 E. L. SMITH, D. M. BROWN AND M. LASKOWSKI, *J. Biol. Chem.*, 191 (1951) 639.
10 M. KUNITZ, *J. Gen. Physiol.*, 30 (1947) 291.
11 M. S. DUNN, *Biochem. Preparations*, 1 (1949) 22.
12 M. L. ANSON, *J. Gen. Physiol.*, 22 (1939) 79.
13 N. M. GREEN AND E. WORK, *Biochem. J.*, 54 (1953) 257.
14 R. E. PARKS, JR. AND G. W. E. PLAUT, *J. Biol. Chem.*, 203 (1953) 755.
15 C. K. KEITH, A. KAZENKO AND M. LASKOWSKI, *J. Biol. Chem.*, 170 (1947) 227.
16 J. A. AMBROSE AND M. LASKOWSKI, *Science*, 115 (1952) 358.
17 L. R. CHRISTENSEN, *Arch. Biochem. Biophys.*, 53 (1954) 128.

Received May 3rd, 1955