Inhibition of Chymotrypsin Activity in Crystalline Trypsin Preparations

VLADIMIR KOSTKA AND FREDERICK H. CARPENTER

From the Department of Biochemistry, University of California, Berkeley, California

(Received for publication, November 18, 1963)

Investigations of protein structures have made extensive use of trypsin to obtain specific cleavage of the peptide chains at the carboxyl groups of lysine and arginine and more recently of aminoethylcysteine residues (1, 2). Unfortunately for such studies, it has been noted that many samples of crystalline trypsin display appreciable amounts of chymotrypsin activity. We have used trypsin to cleave away the carboxyl-terminal octapeptide sequence of the phenylalanyl chain of bovine insulin to give desoctapeptide-insulin (3, 4). In these studies, particular attention was paid to the selection and use of trypsin preparations which were low in chymotryptic activity. Recent investigations have required the use of large amounts of trypsin. Most samples of commercial crystalline trypsin have proved to be unsuitable for the preparation of desoctapeptide-insulin as judged by the following test. The desoctapeptide-insulin was treated with carboxypeptidase A and the amino acids released were determined quantitatively. In theory, only asparagine (or aspartic acid (5)) should be released by the action of carboxypeptidase A on desoctapeptide-insulin. However, samples of desoctapeptide-insulin prepared by the use of most commercial samples of trypsin yielded other amino acids on treatment with carboxypeptidase A. These additional amino acids were primarily tyrosine, phenylalanine, and leucine. Such residues would be liberated by the action of carboxypeptidase A on products arising from chymotryptic cleavage of insulin.

Several procedures have been proposed to decrease or eliminate the chymotryptic activity of trypsin preparations. One involves the treatment of trypsin with dilute acid (6) under conditions designed to decrease the chymotryptic activity without undue damage to the trypsin. This procedure has not been particularly successful in our hands (3). In another procedure the enzyme is treated with diisopropyl fluorophosphate in an amount designed to inhibit all of the chymotrypsin but only partially inactivate the trypsin (7). An exhaustive study of aromatic compounds as inhibitors of $\alpha$-chymotrypsin has been published recently by Wallace, Kurtz, and Niemann (8). Several months ago Schoellmann and Shaw (9) reported on specific labeling of the active center of chymotrypsin with $L$-1-tosylamido-2-phenyl)ethyl chloromethyl ketone; BAE, N'-benzoyl-L-arginine ethyl ester; ATE, N-acetyl-L-tyrosine ethyl ester.

In connection with these studies, several new assays for chymotryptic activity were developed. The spectrophotometric method of Schwert and Takenaka (10) is not very appropriate for the determination of small amounts of chymotryptic activity in trypsin. The assay measures the decrease in absorption at 237 $\mu m$ as the ester bond of N-acetyl-L-tyrosine ethyl ester is cleaved. In order to measure small amounts of chymotryptic impurity, large amounts of trypsin must be added to the cuvette. The resulting change in optical density may be attributed to other factors (such as self-digestion) rather than chymotryptic activity on N-acetyl-L-tyrosine ethyl ester. Further, the intrinsic "N-acetyl-L-tyrosine ethyl ester-cleaving" character of trypsin has been reported (11, 12). Since we were primarily interested in preventing chymotryptic activity on insulin, emphasis was placed on assays in which insulin or its derivatives are used as substrates. In addition to the assay making use of carboxypeptidase A (described above), an assay which used the oxidized B chain of insulin and one that used the heptapeptide, Gly-Phe-Phe-Tyr-Thr-Pro-Lys, as substrates were developed. Use of the oxidized B chain of insulin as a substrate is modeled after the procedure of Crestfield, Moore, and Stein (13), which employs the carboxymethyl derivative of the B chain of insulin as a substrate.

**Experimental Procedure**

**Materials**—Trypsin was a twice crystallized, salt-free, lyophilized sample purchased from Worthington Biochemical Corporation (Lot. 6224). Chymotrypsin was a crystalline, salt-free, lyophilized sample obtained from the same source (Lot. 426-9). Carboxypeptidase A (three times crystallized and treated with diisopropyl fluorophosphatase) was a Worthington preparation (Lot. 6120) secured as an aqueous suspension. Crystalline zinc insulin of bovine origin was purchased from British Drug Houses, Ltd. (Batch 2189) and converted to its hydrochloride before use (4). TPCK $^1$ (m.p. 105-106°) was prepared by the procedure of Schoellmann and Shaw (9). The heptapeptide Gly-Phe-Phe-Tyr-Thr-Pro-Lys (all $L$ isomers) was synthesized earlier in this laboratory by Shields and Carpenter (14). The oxidized B chain of insulin was prepared by the method of Craig, Königsberg, and King (15) and generously furnished by Dr. M. A. Raftery.

$^1$ The abbreviations used are: TPCK, $L$-1-tosylamido-2-phenyl ethyl chloromethyl ketone; BAE, $N'$-benzoyl-$L$-arginine ethyl ester; ATE, N-acetyl-L-tyrosine ethyl ester.
 ion exchange chromatography of tryptic digests of the oxidized B chain of insulin and of controls on the 15-cm column of the amino acid analyzer with 0.35 N sodium citrate, pH 5.28, as eluent. The digests were from: A, TPCK-treated trypsin; B, oxidized B chain of insulin; C, untreated trypsin + the oxidized B chain of insulin; D, TPCK-treated trypsin + the oxidized B chain of insulin; and E, TPCK-treated trypsin + 1% of chymotrypsin + the oxidized B chain of insulin.

Amino Acid Analyses—The amino acid composition of desoctapeptide-insulin and the carboxy-terminal amino acids released by carboxypeptidase A were determined by the method of Spackman, Stein, and Moore (16) with the Spinco/Beckman amino acid analyzer. The complete acid hydrolysates of desoctapeptide-insulin were obtained by heating the sample at 120° in 6 N HCl for 6 hours (17).

Assay A: Activity Measurements—The trypsin activity was determined by measuring the rate of hydrolysis of Nε-benzoyl-L-arginine ethyl ester according to Schwert and Takenaka (10). The measurements were carried out at four different concentrations in a reaction mixture which was 0.00025 M BAE in 0.05 M phosphate buffer at pH 7.0. The decrease in optical density at 253 nm was followed and plotted against the amount of enzyme. The chymotryptic activity was determined by a similar procedure in which 0.001 M ATE was used as substrate in 0.05 M phosphate buffer. The results of chromatography are shown in Fig. 2.

Assay B: Digestion of Oxidized B Chain of Insulin—The oxidized B chain of insulin (7 mg, 2 μmoles) was dissolved in 2 ml of 0.12 N borate buffer at pH 9.4, and 0.75 ml of the trypsin solution corresponding to 0.6 mg (0.024 μmole) of the enzyme was added. The volume was then made up to 3.5 ml with the buffer. Digestion was carried out with TPCK-treated trypsin, with untreated trypsin, and also with a sample of TPCK-treated trypsin which had been made 1% in chymotrypsin by the addition of crystalline commercial chymotrypsin. Solutions of either the B chain alone or TPCK-treated trypsin alone were exposed to the same experimental conditions and served as blanks. The digests were incubated for 5 hours at 37°. The reaction was stopped by the addition of 0.5 ml of 1 N HCl (acidification to pH 1.8), and the reaction mixtures were stored at −10° until analyzed.

To the 15-cm column of the amino acid analyzer were applied 2 ml (1 μmole) of each digest, and the column was eluted with 0.35 N citrate at pH 5.29 for 6 hours at a flow rate of 30 ml per hour. Blank solutions were treated in the same way. The results of chromatography are shown in Fig. 2.

Assay C: Digestion of Heptapeptide—The heptapeptide, Gly-Phe-Phe-Tyr-Thr-Pro-Lys (2.02 mg, 2.2 μmoles), was dissolved in 2.0 ml of 0.12 N borate buffer at pH 9.4. To this solution a volume of an aqueous solution of trypsin corresponding to 0.66 mg (0.024 μmole) of the enzyme was added, and the volume of the reaction mixture was made up to 4 ml with the borate buffer. The experiments were carried out under identical conditions with TPCK-treated trypsin, untreated trypsin, and TPCK-treated trypsin which had been contaminated by the addition of 1% of crystalline chymotrypsin. Samples contain-

Recent studies in this laboratory by S. S. Wang have shown that the amount of TPCK can be decreased by one-tenth, i.e. 29 mg of TPCK per g of trypsin. The product so obtained is as free of chymotryptic activity as the TPCK-treated trypsin used in this report when judged by the assay on the heptapeptide.
ng either only TPCK-treated trypsin without the heptapeptide or the heptapeptide with trypsin served as blanks. All solutions were incubated for 5 hours at 37° with shaking. After this period, 400 µl of 1 N HCl were added to reduce the pH to 1.8, and the digests were stored at -10° until chromatographed.

The 15-cm column of the amino acid analyzer was equilibrated with a pyridine-acetate buffer (0.2 M in pyridine, pH 3.5). After each digest (2 ml, 1 µmole of the heptapeptide) was applied, elution was started by means of a linear gradient developed by mixing a 1 M pyridine-acetate buffer at pH 4.5 with the 0.2 M pyridine-acetate buffer at pH 3.5. The elution was carried out for 12½ hours at a flow rate of 30 ml per hour. Between individual runs the column was washed first with 0.2 M citrate for 2 hours, then regenerated by 0.2 N NaOH, and equilibrated with 0.2 M pyridine-acetate at pH 3.5. The results of chromatography of the digests are summarized in Fig. 3.

Assay D: Digestion of Insulin with Trypsin Followed by Treatment with Carboxypeptidase A—Insulin hydrochloride (90 mg, 10 µmoles) was dissolved in 10 ml of water. The solution was titrated to pH 8.4 with 0.05 N NaOH and was maintained at this pH value by a pH-stat until the alkali uptake achieved a steady slow rate or ceased. TPCK-treated trypsin (1.2 mg, 0.05 µmole in 1 ml) at pH 8.4 was then added, and the alkali uptake was recorded. When the uptake leveled off, the same amount of trypsin was added again. The addition of trypsin was repeated three more times. The final amount of TPCK-treated trypsin in the digest was 6.0 mg (enzyme to substrate weight ratio, 1:10). The digestion was stopped after 5 to 6 hours by the addition of 0.1 N HCl to pH 4.7. The digest was set aside overnight at 4°, and the precipitated desoctapeptide-insulin was isolated by centrifugation. The precipitate was washed three times with water at pH 4.7, twice with acetone, and once with U.S.P. ether from a newly opened can, and dried overnight in an evacuated desiccator over phosphorus pentoxide. The yield of desoctapeptide-insulin was about 70%. The supernatant after the first centrifugation of desoctapeptide-insulin was adjusted to pH 3, and the solution was saved for trypsin activity assays.

Desoctapeptide-insulin (6.2 mg, 1.24 µmoles) was dissolved in 1.5 ml of 0.05 M Tris buffer at pH 7.4 (5), and the pH was adjusted to 7.4 by the addition of 0.1 N NaOH. A solution (20 µl) of carboxypeptidase A corresponding to 0.25 mg of the enzyme (enzyme to substrate weight ratio, 1:25) was added together with 50 µl of toluene, which served as a preservative. The solution was then incubated at 37° for 4 hours. After this period, 10 µl of the enzyme solution (0.125 mg; weight ratio, 1:50; total enzyme to substrate weight ratio, 1:16) were added, and the digestion was continued for 4 hours. The reaction was stopped by adjusting the volume of the reaction mixture to 5 ml with 0.1 N HCl to decrease the pH to 2. The digest was kept frozen until analyzed on the 50-cm column of the amino acid analyzer (5). A blank solution containing only the enzyme was subjected to the same treatment.

RESULTS AND DISCUSSION

Data of Schoellmann and Shaw (9) on the pH dependence of the inactivation of chymotrypsin by TPCK indicate that the reaction reaches its maximum rate at approximately pH 7. These results were confirmed in our preliminary experiments with crystalline chymotrypsin. After a 3 X 10^{-6} M solution of chymotrypsin in 0.01 M phosphate at pH 6.0 had been incubated with a 20-fold molar excess of TPCK for 2½ hours, its activity was found to be 20% of a control sample. When the reaction was carried out at pH 7.0, the activity of TPCK-treated chymotrypsin was zero, and only a slight drop in the activity of the control sample was observed (7%). Commercial trypsin was exposed to the same conditions as chymotrypsin at pH 7. After the treatment, both the trypsinic and chymotryptic activity of the product were investigated by the spectrophotometric

Fig. 3. Ion exchange chromatography of tryptic digests of the heptapeptide, Gly-Phe-Phe-Tyr-Thr-Pro-Lys, and of controls on the 15-cm column of the amino acid analyzer with a linear gradient of pyridine-acetate buffers. The digests were from: A, TPCK-treated trypsin; B, the heptapeptide; C, untreated trypsin + the heptapeptide; D, TPCK-treated trypsin + the heptapeptide; and E, TPCK-treated trypsin + 1% of chymotrypsin + the heptapeptide.

The use of pyridine-containing buffers usually results in the swelling of the resin particles, which makes the subsequent regeneration both difficult and time-consuming. The time necessary for the regeneration can be shortened if the resin is washed first for approximately 120 minutes with 0.2 N sodium citrate at pH 3.5.
Table I

<table>
<thead>
<tr>
<th>Release of amino acids during action of carboxypeptidase A on desoctapeptide-insulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amino acids released by carboxypeptidase A</td>
</tr>
<tr>
<td>Leucine</td>
</tr>
<tr>
<td>moles/mole asparagine + asparic acid</td>
</tr>
<tr>
<td>Commercial trypsin</td>
</tr>
<tr>
<td>TPCK-trypsin</td>
</tr>
</tbody>
</table>

When compared with the activity of a control sample, the tryptic activity of TPCK-treated trypsin was found to be essentially the same (93 to 100%). At the same time the chymotryptic activity of TPCK-treated trypsin (Fig. 1) dropped by approximately 50% (from 0.46 to 0.25%) as measured on ATE.

In the preliminary work, the trypsin was in a very dilute solution (0.075 mg per ml) when treated with TPCK. In order to treat gram quantities of trypsin under these conditions, an awkwardly large reaction volume would be involved, especially at the dialysis and lyophilization steps. Therefore, the concentration of trypsin was raised from $3 \times 10^{-6}$ to $1.2 \times 10^{-4}$ M. The same molar excess of the inhibitor (20-fold) was used. In place of the phosphate buffer, the pH-stat was used to maintain the reaction mixture at a constant value of pH 7. The reaction time was prolonged to 5 hours, and the alkali uptake was recorded. The uptake was approximately 50% higher than when trypsin was treated under the same conditions in the absence of the inhibitor.

**Assay A: Chymotryptic Activity of TPCK-Treated Trypsin as Measured on Acetyl-l-tirosine Ethyl Ester—**Chymotryptic activity of TPCK-treated trypsin was tested on ATE. The results were essentially the same as those obtained in the preliminary experiments (cf. Fig. 1). It was observed that the value for the chymotryptic contamination varied with the amount of trypsin analyzed. However, the chymotryptic activity of the TPCK-treated trypsin was decreased to about 50% of that of the untreated trypsin when the two were analyzed at the same level of protein.

**Assay B: Chymotryptic Activity as Measured on Oxidized B Chain of Insulin—**The oxidized B chain of insulin was treated with the various trypsin preparations, and the products formed were detected by ion exchange chromatography. The conditions were similar to those proposed by Crestfield et al. (13) for detection of chymotryptic activity with the carboxymethyl derivative of the B chain of insulin as a substrate. The action of trypsin on the oxidized B chain of insulin should yield alanine, a heptapeptide (Gly-Phe-Phe-Tyr-Thr-Pro-Lys), and the remaining portion of the chain. Previous experience (4) with the 15-cm column and 0.35 N citrate (pH 5.28) as eluent had shown that the alanine would move off near the solvent front while the heptapeptide would be bound to the resin unless a pyridine buffer was applied. Curves A of Fig. 2 show the products found in the control sample of TPCK-treated trypsin which had been incubated in the absence of substrate. There is a minor peak at the solvent front, an ammonia peak at 105 ml (which occurs in all the samples), and a small peak at 120 ml. Chromatography of the oxidized B chain of insulin which had been incubated in the absence of enzyme (Curves B, Fig. 2) shows a minor peak at the solvent front, a broad peak at 30 ml which can be attributed to the acidic substrate, the ammonia peak at 105 ml, and a small peak at 120 ml. A very similar result was obtained when the products formed by the action of TPCK-treated trypsin on the substrate were chromatographed (Curves D, Fig. 2). The peak at 30 ml had largely disappeared and had been replaced by a large peak at 15 ml which can be attributed to alanine and also possibly to the desoctapeptide portion of the B chain. In any event, quite a different result was obtained when the products formed by the action of untreated trypsin were subjected to chromatography (Curves C, Fig. 2). Several new peaks, one at 65 ml of effluent, a doublet at 75 ml, and one at 135 ml of effluent, were observed. The results found with the untreated trypsin were almost identical with those found for the TPCK-treated trypsin which had been contaminated with 1% chymotrypsin (compare Curves C and E of Fig. 1). These facts strongly suggest that the peaks found in the digest which used untreated trypsin and those which are absent in the digest which used TPCK treated trypsin are formed by the action of chymotrypsin on the substrate. Furthermore, the results indicate the absence of such chymotryptic activity in the TPCK-treated trypsin.

**Assay C: Chymotryptic Activity as Measured on Heptapeptide—**The synthetic heptapeptide, Gly-Phe-Phe-Tyr-Thr-Pro-Lys, does not contain any bonds susceptible to the action of trypsin, but does contain a number of bonds which are susceptible to the action of chymotrypsin. Consequently, it should be a very good substrate to test for the presence of chymotrypsin in trypsin preparations. The heptapeptide was digested with TPCK-treated trypsin, with untreated trypsin, and with TPCK-treated trypsin artificially contaminated with 1% of crystalline chymotrypsin. The digestions were carried out under conditions similar to those used for the digestion of the oxidized B chain of insulin. For the chromatographic separation of the products of the digests, a linear elution gradient of pyridine-acetate buffers was found to be more effective than the system described for the separation of the heptapeptide itself by Carpenter and Baum (4). The results of chromatography are summarized in Fig. 3. The sample from the incubation of TPCK-treated trypsin with no substrate does not contain any significant peaks (Curves A, Fig. 3). Chromatography of the heptapeptide reveals a single peak which emerges at 352 ml (Curves B, Fig. 3). The results of chromatography of the digests obtained from the action of TPCK-treated trypsin on the heptapeptide (Curves D, Fig. 3) are essentially identical with those obtained for the heptapeptide alone. Furthermore, the color yield of the heptapeptide peak was practically identical in the two cases. In sharp contrast to these results, chromatography of the products formed by the action of untreated trypsin on the heptapeptide revealed a number of new peaks and a decrease in the amount of material in the heptapeptide peak (Curves C, Fig. 3). These latter results were almost identical with those obtained upon chromatography of the digests of the heptapeptide by TPCK-treated trypsin which had been made 1% in chymotrypsin (compare Curves C and E of Fig. 3). These results show in an unequivocal manner that the untreated trypsin was contaminated with an enzyme possessing the same specificity as chymotrypsin and that this extra enzyme activity could be completely inhibited by treatment of the trypsin preparation with TPCK.

**Assay D: Chymotryptic Activity as Measured on Insulin—**It
has been shown (3) that the action of trypsin on insulin results in the formation of desoctapeptide-insulin, free alanine, and the heptapeptide. Desoctapeptide-insulin comprises two chains, carboxyl-terminated with asparagine and arginine, respectively. The action of carboxypeptidase A on desoctapeptide-insulin should, therefore, liberate only carboxyl-terminal asparagine (and aspartic acid (5)) from the A chain, whereas the arginine residue of the B chain should not be released. The presence of any amino acid other than asparagine (or aspartic acid) in the digest from the action of carboxypeptidase A on desoctapeptide-insulin would indicate additional nonspecific cleavage of the peptide chains by the trypsin preparation used to prepare the desoctapeptide-insulin. In this study, TPCK-treated trypsin was used for the digestion of insulin, and the isolated desoctapeptide-insulin was treated with carboxypeptidase A (5). The carboxypeptidase digest was analyzed for free amino acids. The results were compared with those obtained from the carboxypeptidase A digestion of desoctapeptide-insulin prepared by the use of untreated trypsin. The values are given in Table I. It may be seen that the amounts of tyrosine, leucine, and phenylalanine found in the digestive of the desoctapeptide-insulin prepared by use of TPCK-treated trypsin are considerably lower than the amounts found in digests of desoctapeptide-insulin prepared by use of untreated trypsin. The fact that desoctapeptide-insulin prepared by use of untreated trypsin released leucine, tyrosine, and phenylalanine in addition to asparagine and aspartic acid on treatment with carboxypeptidase A is a strong indication that the desoctapeptide-insulin contained some cleavages in the peptide chain brought about by the action of chymotrypsin. The very small amounts of leucine, tyrosine, and phenylalanine found in the carboxypeptidase A digests of desoctapeptide-insulin prepared by use of TPCK-treated trypsin indicate the virtual elimination of contaminating chymotryptic activity in TPCK-treated trypsin.

SUMMARY

The use of the chymotrypsin inhibitor, L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone (TPCK), to inhibit the chymotrypsin activity of commercial samples of crystalline trypsin has been investigated. The TPCK-treated trypsin, the untreated trypsin, and TPCK-treated trypsin which had been made 1% in chymotrypsin were tested for chymotryptic activity in four independent assays: Assay A, spectrophotometric assay on acetyl-l-tyrosine ethyl ester; B, digestion of the oxidized B chain of insulin, followed by ion exchange chromatographic separation of the products; C, treatment of the heptapeptide Gly-Phe-Phe-Tyr-Thr-Pro-Lys, followed by ion exchange chromatography of the products; and D, digestion of insulin, followed by carboxypeptidase A hydrolysis of the desoctapeptide-insulin and chromatographic detection of the released amino acids. As compared with the untreated sample of trypsin, the chymotryptic activity of TPCK-treated trypsin was either greatly diminished (Assay A) or virtually eliminated (Assays B to D).

Acknowledgments—The authors wish to thank Mrs. Bertha L. Martin for her help with the amino acid analyses, and Dr. M. A. Raftery for supplying the oxidized B chain of insulin.

REFERENCES

Inhibition of Chymotrypsin Activity in Crystalline Trypsin Preparations
Vladimir Kostka and Frederick H. Carpenter


Access the most updated version of this article at
http://www.jbc.org/content/239/6/1799.citation

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at
http://www.jbc.org/content/239/6/1799.citation.full.html#ref-list-1