Enzymic and Physicochemical Properties of *Streptomyces griseus* Trypsin†

Robert W. Olafson‡ and Lawrence B. Smillie*

ABSTRACT: *Streptomyces griseus* trypsin has been isolated from Pronase by ion-exchange chromatography on CM-Sephadex and SE-Sephadex. The isolated enzyme was homogeneous by the criteria tested except for a low degree of contamination by an enzyme with nontryptic activity. The latter could be partially resolved by chromatography on Bio-Rex 70. The molar absorbancy at 280 nm was found to be 3.96×10^4 M^{-1}/cm and the $E_{1cm}^{1\%}$ was found to be 17.3. The molecular weight was 22,800 ± 800. The enzyme was found to be stable at 0° from pH 2 to 10. At 30° the enzyme was maximally stable at pH 3–4 and significantly stabilized in the neutral and alkaline range by 15 mM Ca^{2+}. Some evidence was obtained for a reversible denaturation of the enzyme at pH 12.0 and 2.0. The K_m for *N*-α-benzoyl-L-arginine ethyl ester at pH 8.0 in 20 mM $CaCl_2$–0.1 M KCl–10 mM Tris-HCl buffer at 30° was found to be 7.7 ± 1.9×10^6 M and the esterase activity was observed to be dependent on an ionizing group with $pK_a = 5.85$. In ^2H_2O this pK_a was increased to 6.35 and the rate of hydrolysis decreased threefold. The rate of hydrolysis was independent of pH between 8 and 10. The inhibition of the enzyme with L-1-chloro-3-tosylamido-4-phenyl-2-butanone was shown to be associated with the alkylation of its single histidine residue. This residue is present in a homologous amino acid sequence as the active-site histidine in trypsin and chymotrypsin. Optical rotatory dispersion and circular dichroism measurements over the pH range 5.3–10.5 indicated no significant conformational change until the pH was increased above 10.1. The observation that, under the conditions tested, acetylation and carbamylation of the NH_2-terminal valine were incomplete is consistent with the view that this group is buried as an ion pair and only becomes available for deprotonation and reaction upon denaturation of the enzyme at pH values greater than 10.0.

Trypsin and trypsin-like enzymes have been isolated from a number of mammalian sources as well as from dogfish, starfish, and horseshoe crab (Walsh, 1970), shrimp (Gates and Travis, 1969), sea pansy (Coan and Travis, 1970), and from silkworms (Kafatos *et al.*, 1967). It is now apparent that trypsin also occurs in certain microorganisms including several species of *Streptomyces* (Wählby *et al.*, 1965; Morihara and Tsuzuki, 1968; Yoshida *et al.*, 1971). The most thoroughly studied of these has been *Streptomyces griseus* trypsin (S.G. trypsin[1]) isolated from the commercial product Pronase, a mixture of enzymes and proteins derived from the extracellular filtrate of cultures of the K-1 strain of this microorganism. This enzyme has been shown to hydrolyze BzArgOEt[1] (Wählby, 1968), to be specific for the hydrolysis of lysine and arginine residues in the β chain of oxidized insulin (Jurášek *et al.*, 1969), and to be inhibited by iPr_2P-F[1] (Wählby and Engström, 1968; Trop and Birk, 1968), Tos-LysCH_2Cl (Yoshida *et al.*, 1971), and by naturally occurring trypsin inhibitors (Birk, 1968; Trop and Birk, 1971). The enzyme was shown to have the active serine sequence Asp-Ser-Gly (Wählby and Engström, 1968) and to have a remarkable homology with bovine trypsin in the amino acid sequence about its three disulfide bridges (Jurášek *et al.*, 1969).

Because of the widely different phylogenetic sources of this enzyme and bovine trypsin, it was of considerable interest to more fully characterize the *Streptomyces griseus* enzyme with respect to its enzymic and physicochemical properties. The results of these studies are presented in the present paper which have shown that by a number of criteria the two enzymes are very similar indeed. In the accompanying paper (Olafson *et al.*, 1975) it is shown that these similarities can be attributed to a high degree of homology in their amino acid sequences and undoubtedly in the conformations of their polypeptide chains.

Experimental Procedure

Materials. Pronase (B grade) was purchased from Calbiochem, Los Angeles, Calif. CM-Sephadex C-50 and SE-Sephadex C-50 were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. *N*-α-Benzoyl-L-arginine ethyl ester, oxidized insulin A and B chains, and cacodylic acid were all purchased from Schwarz/Mann Chemical Co., New York, N.Y. Deuterium oxide was a gift from Dr. M. Barton, Department of Biochemistry, University of Alberta. Tos-LysCH_2Cl and *N*-aminobenzamidine were purchased from Sigma Chemical Co., St. Louis, Mo.

Methods. Pronase was initially fractionated using the CM-Sephadex procedure as described by Jurášek *et al.* (1971). The pooled S.G. trypsin fraction was then further purified by chromatography on Bio-Rex 70 (Jurášek *et al.*, 1969) or on SE-Sephadex C-50. For the latter procedure, the dialyzed and lyophilized S.G. trypsin fraction (800 mg)

† From the Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada. *Received May 22, 1974.* Taken, in part, from a dissertation submitted by R.W.O. to the Faculty of Graduate Studies of the University of Alberta in partial fulfillment of the requirements for the degree of Doctor of Philosophy. This investigation was supported by a grant from the Medical Research Council of Canada. R.W.O. was supported by a Medical Research Council of Canada Studentship (1969–1972).

‡ Present address: Institute of Oceanography, University of British Columbia, Vancouver 8, B.C.

[1] Abbreviations used are: S.G. trypsin, *Streptomyces griseus* trypsin; iPr_2P-F, diisopropyl phosphorofluoridate; Tos-LysCH_2Cl, L-1-chloro-3-tosylamido-7-amino-2-heptanone; Tos-PheCH_2Cl, L-1-chloro-3-tosylamido-4-phenyl-2-butanone; BzArgOEt, *N*-α-benzoyl-L-arginine ethyl ester; AcTyrOEt, *N*-acetyl-L-tyrosine ethyl ester; ORD, optical rotatory dispersion; CD, circular dichroism.

was dissolved in 0.1 M pyridine–acetic acid buffer (pH 5.0) and applied to a 5 × 100 cm column previously equilibrated with the same buffer at 5°. A linear gradient to 0.5 M pyridine–acetate (pH 5.0) was established with 4.3 l. of buffer in each of the two reservoirs of the gradient device. The pooled S. G. trypsin fractions, detected by their absorbance at 280 nm and their *N*-α-benzoyl-L-arginine ethyl esterase activities, were lyophilized directly. In some cases, for the preparation of highly purified enzyme for enzymic specificity studies, the S.G. trypsin was isolated by ion-exchange chromatography on all three systems in the order: CM-Sephadex, SE-Sephadex, and Bio-Rex-70.

Esterase Activities. *N*-α-Benzoyl-L-arginine ethyl esterase activities were measured in the pH-Stat at 25° with 10 mM BzArgOEt in 20 mM $CaCl_2$–0.1 M KCl–10 mM Tris-HCl (pH 8.0). One enzyme unit was that activity which would hydrolyze 1 μmol of substrate/min. Specific activity was defined as the units of enzyme activity per milligram of protein. These varied for different S.G. trypsin preparations from 150 to 170 units/mg. For studies of the effect of pH on activity the assays were performed in the pH-Stat as described above except that each assay solution was adjusted to the desired pH with 0.2 N NaOH or 0.2 N HCl. In the heavy water experiments the pH[2] was assumed to be the measured pH + 0.40 (Glasoe and Long, 1960). For the determination of K_m values the assays were performed with 1-l. volumes of substrate in the same buffer as above under a nitrogen atmosphere. The BzArgOEt concentrations were varied from 20 to 1.54 μM and the temperature was maintained at 30° with a circulating water bath. Values were calculated from a Lineweaver–Burk plot in the usual way.

Ultracentrifugation. Molecular weight determinations were performed with a Beckman Model E ultracentrifuge employing the Rayleigh interference optical system and the low-speed methodology of Richards *et al.* (1968). Overspeed was 26,000 rpm for 3 hr followed by an equilibrium speed of 15,000 rpm for 18 hr. The protein concentration was 1 mg/ml in 0.5 M KCl–0.05 M acetate buffer (pH 5.0) and the temperature was 20°. Molecular weights were computed using an IBM 360 computer with programs made available to us by Dr. W. T. Wolodko. A value of 0.717 ml/g for the partial specific volume of S.G. trypsin was assumed (Travis and Roberts, 1969).

Stability studies. The stability of S.G. trypsin was examined over the pH range 1.0–11.0 using the following solutions: pH 1.0–2.0, 10 mM KCl–HCl; pH 2.5–3.5, 10 mM sodium formate–formic acid; pH 4.0–5.0, 10 mM sodium acetate–acetic acid; pH 6.0–8.0, 10 mM Tris-maleate; pH 9.0–12.0, 50 mM glycine–sodium hydroxide. The protein concentration was 0.2 mg/ml and the calcium concentration when present was 15 mM. Following incubation at 0° or 30°, BzArgOEtase activity was measured in the usual way.

Inhibition with Tos-LysCH_2Cl. To follow the rate of inhibition with Tos-LysCH_2Cl, the enzyme (2.5 mg, 0.11 μmol) was dissolved in 1 ml of 50 mM Tris-maleate (pH 7.0) containing 5 mM $CaCl_2$. The reaction was initiated by adding an equal volume of the same buffer containing 0.25 mg (0.75 μmol) of inhibitor. The mixture was incubated at 30° and samples taken for BzArgOEtase assays with time. For studies of the effect of a competitive inhibitor on the reaction, *p*-aminobenzamidine was added to the buffer at a concentration of 0.66 mg/ml (4.88 μmol/ml). For the preparation of Tos-LysCH_2Cl inhibited S.G. trypsin in quantities sufficient for chemical characterization, the procedure described by Shaw (1967) was used precisely as described.

Characterization of alkylated histidine peptide. Tos-LysCH_2Cl-inhibited S.G. trypsin was digested with pepsin and subjected to the cysteic acid diagonal procedure of Brown and Hartley (1966) as applied to the enzyme by Jurášek *et al.* (1969). A control digest and diagonal were carried out with native S.G. trypsin. Isolation of peptides, staining reactions, and procedures for amino acid and NH_2-terminal analyses are described elsewhere (Olafson *et al.*, 1975).

Optical rotatory dispersion (ORD)[1] and circular dichroism (CD)[1] A Cary Model 60 recording spectropolarimeter, equipped with a Cary Model 6001 CD attachment and water cooled lamp housing, was used for these measurements in accordance with the methodology described by Kay and Oikawa (1966) and Oikawa *et al.* (1968). Samples of S.G. trypsin (0.7 mg/ml) were subjected to visible (300–550 nm) and ultraviolet (200–250 nm) ORD scans at pH 5.0–10.5 at 10°. The solvent system was 0.05 M KCl titrated to the desired pH with 0.1 N HCl or 0.1 N NaOH. All solutions with pH values greater than 8.0 contained 20 mM glycine. CD scans (190–250 nm) used the same solvent system but protein concentrations were 0.2 mg/ml. In ORD experiments the α-helix content was calculated for each pH value studied using the Moffit equation (Moffitt and Yang, 1956). Data computed in the usual manner, with $[m'](\lambda^2 - \lambda_0^2)/\lambda_0^2$ plotted on the ordinate *vs.* $\lambda_0^2/(\lambda^2 - \lambda_0^2)$ on the abscissa, were used to evaluate protein solutions at each pH studied with respect to b_0, the slope, and a_0, the intercept. The value λ_0 was taken as 212 nm. For CD measurements the mean residue ellipticity is given by: $[\theta] = \theta^\circ M/100lc$, where M is the mean residue molecular weight, θ° is the observed ellipticity in degrees, l is cell path length in decimeters, and c is the protein concentration in grams per cubic centimeter. The units of $[\theta]$ are (deg cm^2)/dmol.

Acetylation and carbamylation of S.G. trypsin. The procedure of Oppenheimer *et al.* (1966) was followed except that the concentration of S.G. trypsin was 3.0 mg/ml. The resulting opalescent mixture was dialyzed overnight against 4 l. of dilute acetic acid (pH 4.0), the precipitate removed by centrifugation, and the supernatant lyophilized. For carbamylation, 0.4 g of KCNO was added to 10 mg of the enzyme dissolved in 5 ml of water at 0° in the pH-Stat. The pH was maintained at 7.0 by automatic titration with 6 N HCl. After 150 min the soluble product was dialyzed against 50 mM acetic acid (pH 3.5) and lyophilized.

Results

Homogeneity. S.G. trypsin was isolated in this work by an initial fractionation of Pronase on CM-Sephadex (Jurášek *et al.*, 1971) followed by ion-exchange chromatography on either SE-Sephadex C-50 or Bio-Rex 70. The elution profile of pooled S.G. trypsin fractions from a CM-Sephadex separation which were applied to the SE-Sephadex column is shown in Figure 1. Fractions containing BzArgOEtase activity were pooled as indicated and lyophilized without dialysis. When a portion of this material was chromatographed on a Bio-Rex 70 column, a single symmetrical peak was obtained (Figure 2) with only an indication of minor contamination possibly arising from autolysis of the enzyme. When a portion of this same material was inhibited with Tos-LysCH_2Cl and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis under the conditions of Weber and Osborne (1969), a single homogeneous band was observed. Similarily, material isolated from Pro-

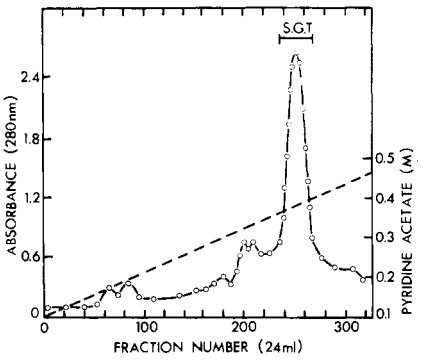

FIGURE 1: SE-Sephadex chromatography of 800 mg of S.G. trypsin previously isolated on CM-Sephadex. Column size 5 × 100 cm. Column equilibrated with 0.1 M pyridinium acetate (pH 5.0) and developed with a linear gradient (- -) increasing to 0.5 M pyridinium acetate at the same pH; (○) absorbancy at 280 nm; flow rate, 100 ml/hr.

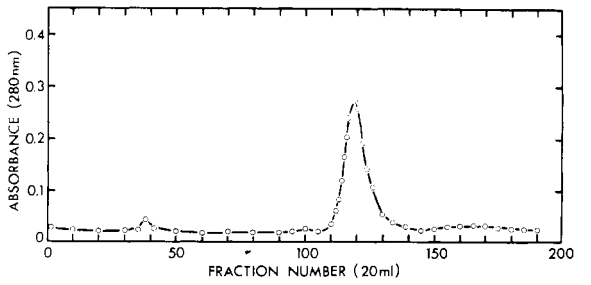

FIGURE 2: Bio-Rex 70 chromatography of 100 mg of S.G. trypsin previously purified by ion-exchange chromatography on CM-Sephadex and SE-Sephadex. The resin was equilibrated with 0.1 N NaOH cacodylate buffer (pH 6.10): column size, 5 × 75 cm; flow rate, 60 ml/hr.

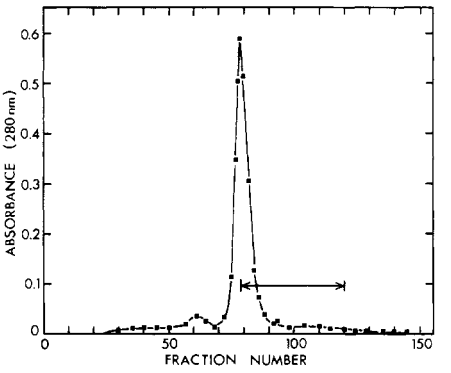

FIGURE 3: Bio-Rex 70 chromatography of 75 mg of S.G. trypsin previously purified on CM-Sephadex and SE-Sephadex. The conditions were as in Figure 2 except that the column was 2.5 × 160 cm and the flow rate 18 ml/hr. The double-headed arrow indicates those fractions containing nontryptic proteolytic activity as indicated by their hydrolysis of the oxidized A chain of insulin.

nase by CM-Sephadex and Bio-Rex 70 chromatography appeared to be equally homogeneous by these criteria. Further evidence for the chemical purity of the S.G. trypsin preparations has come from amino acid sequence analysis as described in the accompanying paper (Olafson *et al.*, 1975) where no peptides which could not be accounted for by the final sequence were isolated from several types of proteolytic digestions of the protein.

Substrate Specificity of S.G. Trypsin. Jurášek *et al.* (1969) showed that highly purified S.G. trypsin appeared to be specific in its hydrolysis at the lysine and arginine residues of the B chain of oxidized insulin but had no activity toward the A chain of insulin. In these experiments the S.G. trypsin had been purified on CM-cellulose (Wählby and Engström, 1968) followed by two chromatographic purification steps on Bio-Rex 70. The digestion conditions were a 1:200 molar ratio of S.G. trypsin to insulin chain at 30° for 1 hr at pH 8.0.

In the present work using S.G. trypsin purified on CM-Sephadex followed by a single chromatographic step on either SE-Sephadex or Bio-Rex 70, we have consistently observed proteolytic activity toward the oxidized A chain of insulin indicating the presence of a small amount of nontryptic activity not detectable by the homogeneity criteria described above. At a molar ratio of 1:20, for example, the preparations hydrolyzed both the A and B chains of insulin at valines, tyrosines, and leucines in addition to the expected cleavages at the arginine and lysine residues present in the B chain. At lower ratios (1:1000) these nonspecific cleavages were confined to a single cleavage in the A chain at tyrosine-14. That this nontryptic activity in our S.G. trypsin preparations was present as a minor enzymic contaminant and not as an inherent activity of S.G. trypsin was demonstrated in the following ways. S.G. trypsin, isolated by CM-Sephadex and SE-Sephadex chromatography as described above, was chromatographed on a Bio-Rex 70 column as previously described (Jurášek *et al.*, 1969). Samples were taken across the peak (Figure 3) and added to oxidized insulin A chain to give a final enzyme to substrate molar ratio of 1:250. Digestions were for 2.5 hr at 25°, at pH 7.5 in 50 mM *N*-ethylmorpholine acetate. The presence of nontryptic A-chain splitting activity was qualitatively detected by examination of the digests by paper high-voltage electrophoresis and staining with cadmium-ninhydrin reagent. The presence of such activity was found in the fractions indicated by the double-headed arrow in Figure 3. It is clear that this nontryptic activity was partially resolved from the main S.G. trypsin peak and appeared to be associated with a low level of absorbance eluting just behind the major S.G. trypsin component. Further evidence for the separate identity of this nontryptic activity was obtained from a comparison of the action of S.G. trypsin and of Tos-LysCH_2Cl-inhibited S.G. trypsin on the two oxidized insulin chains. It was observed that whereas the activity toward the B chain of insulin was essentially completely inhibited by treatment of S.G. trypsin with Tos-LysCH_2Cl, the A-chain splitting activity was not significantly affected. These results are all consistent with our previous conclusion that the intrinsic activity of S.G. trypsin is directed only toward peptide bonds involving lysine and arginine residues. The results also indicate that S.G. trypsin prepared by the methods described in the present work is homogeneous except for a minor nontryptic enzymic contaminant only detectable by enzymic assays.

Molar Absorbancy and Molecular Weight. The molar absorbancy of S.G. trypsin in water at 4° was estimated from measurements of the absorbance at 280 nm and from estimations of the concentration of the same solution by amino acid analyses. For this calculation the molecular weight was taken as 22,918 (see below) and the concentration calculated assuming an alanine and leucine content of 28 and 12 residues, respectively (Olafson and Smillie, 1975). ϵ was found to be 3.96×10^4 M^{-1}/cm and $E_{1cm}^{1\%}$ to be 17.3. The molecular weight of S.G. trypsin was estimated by use of the low-speed sedimentation equilibrium technique. The plots of ln y *vs.* r^2 were linear. The results of two independent runs gave an average molecular weight of 22,800 ± 800, a value in excellent agreement with the mini-

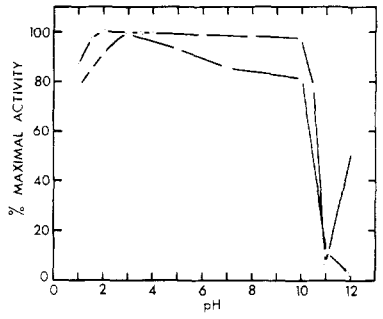

FIGURE 4: Effect of pH on the stability of S.G. trypsin (0.2 mg/ml) at 30°: (●) 25 min in the presence of 15 mM Ca^{2+}; (△) 25 min in the absence of Ca^{2+}; (○) 24 hr in the absence of Ca^{2+}.

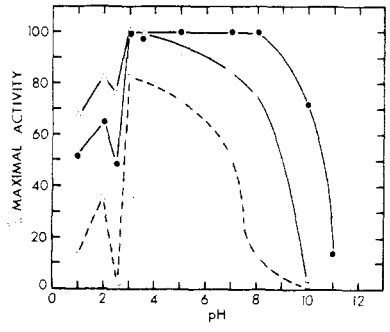

FIGURE 5: Effect of pH on the stability of S.G. trypsin (0.2 mg/ml) at 30°: (●) 25 min in the presence of 15 mM Ca^{2+}; (△) 25 min in the absence of Ca^{2+}; (○) 24 hr in the absence of Ca^{2+}.

mum molecular weight of 22,918 calculated from the amino acid sequence (Olafson *et al.*, 1975).

Stability of S.G. Trypsin. The effects of pH and Ca^{2+} on the stability of the protein at 0 and 30° were carried out for varying times. At 0° the enzymic activity is very stable from pH 2 to 10 (Figure 4) and even after 12 hr the loss in activity at pH 1.0 and 10.0 was only about 20%. Between pH 10.0 and 11.0 there is a dramatic decrease in activity even at very short incubation times. Interestingly the rate of loss of activity at pH 12.0 is slower than at pH 11.0 since some 50% of the activity remained after 60 min of incubation under these conditions.

Not unexpectedly the enzyme is less stable at 30° (Figure 5). Thus, at pH 8.0 the activity has decreased to 80% of its original value after 25 min and to 10% after 24 hr. At pH 10.0 after both 25 min and 24 hr the activity decreased essentially to zero. Ca^{2+}, however, confers a significant degree of stabilization in the neutral and alkaline range since from pH 5.0 to 8.0 there was no loss of activity after 25 min at 30° when 15 mM Ca^{2+} was present. Interestingly at pH 2.0, the rate of loss of activity was slower than at pH 2.5, a phenomenon similar to that observed at pH 12.0 when compared with pH 11.0 at 0°. These observations are most readily explained by assuming that there is a reversible equilibrium between native and denatured enzyme which at the less extreme pH values results in the autolysis of the denatured form. However, at more extreme pH values, *i.e.* pH 2.0 and 12.0, the concentration of the native enzyme becomes so small and its activity so low that the reversibly denatured form accumulates. Further exposure to these conditions leads to irreversible denaturation but the reversibly denatured form may be converted to the active enzyme under the assay conditions at pH 8.0. Although this phenomenon has not been investigated in detail it has been observed that in the assay of the S.G. trypsin incubated at pH 2.0 and 12.0, activity rapidly increased for the first few seconds subsequent to mixing enzyme with substrate. These observations are consistent with a rapid conversion of the reversibly denatured enzyme to the active form under these conditions. Similar observations have been made with bovine trypsin by a number of workers (Green and Neurath, 1954).

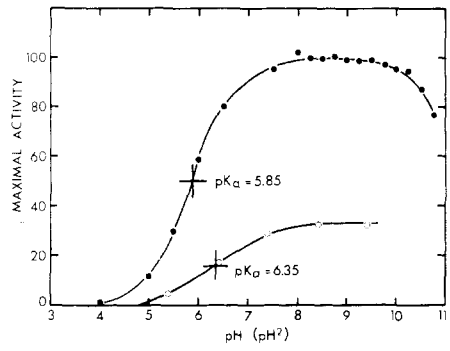

FIGURE 6: Effect of pH on the activity of S.G. trypsin in ^2H_2O (●) and in ^2H_2O (○).

Michaelis Constant for BzArgOEt Hydrolysis. The average of five determinations of K_m, measured at 30° over a substrate concentration range of 1.54×10^{-6} to 20.0×10^{-6} M, was $7.7 \pm 1.9 \times 10^{-6}$ M. This is in excellent agreement with the K_m of 7.3×10^{-6} M reported by Yoshida *et al.* (1971) for S.G. trypsin and with the value of 1×10^{-5} M for bovine trypsin (Gutfreund, 1955).

Effects of pH and p^{2}H on BzArgOEtase Activity. The activity of S.G. trypsin (Figure 6) was measured over the pH range 3-10.5. It is clear that the activity appears to be associated with the deprotonation of a group having a pK_a of 5.85. This value was the average of four determinations ranging from 5.70 to 5.95 and is in reasonable agreement with the value of 6.25 for bovine trypsin reported by Gutfreund (1955). In the case of bovine trypsin and other serine proteases this pK_a has been associated with the ionization of the imidazole of histidine-57 (chymotrypsinogen numbering scheme) although recent evidence from nuclear magnetic resonance studies of Myxobacter α-lytic protease indicates that it should in fact be assigned to aspartate-102 in the active-site triad aspartate-102, histidine-57, serine-195 (Hunkapiller *et al.*, 1973). In any case the somewhat lower value in the case of S.G. trypsin indicates that the microenvironment in the region of the active site may be somewhat different than in bovine trypsin.

The BzArgOEtase activity in ^2H_2O, plotted as a function of p^{2}H is also shown in Figure 6. It is seen that when water is replaced by H_2O_2, the pK_a shifts from 5.85 to 6.35 and the hydrolysis rate is reduced threefold. In this respect S.G. trypsin behaves similarily to the other serine proteases (Bender and Hamilton, 1962; Kaplan and Whitaker, 1969) for which this effect has been described as being consistent with general basic catalysis by an imidazole group (Bender *et al.*, 1962). Although this interpretation may be questioned (Gutfreund and Knowles, 1967) it is significant that S.G. trypsin exhibits the same phenomena in ^2H_2O as do other well-characterized serine proteases.

A further significant feature of the activity of S.G. trypsin is that the rate of hydrolysis becomes independent of pH when the pH exceeds 8.0 and only shows a significant decrease above a pH of 10.0 where the enzyme becomes un-

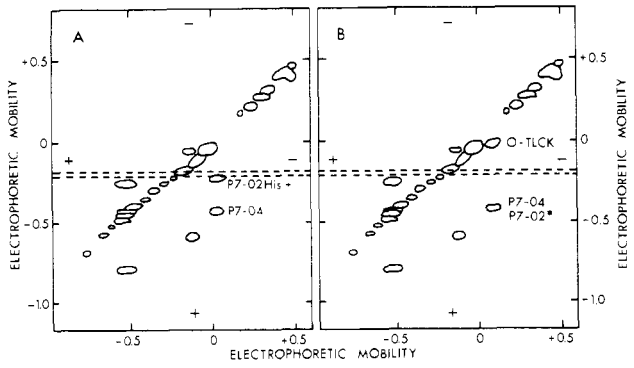

FIGURE 7: Peptide diagonal maps (pH 6.5/pH 6.5) of peptic digests of native S.G. trypsin (A) and of Tos-LysCH_2Cl-inhibited S.G. trypsin (B). The Pauly-positive peptide P7-02 present in A is replaced by Pauly-negative peptide P7-02* in B which migrates in the same position as P7-04. Spot O-TLCK contains no peptide material and is an oxidative degradation product of Tos-LysCH_2Cl.

stable. Thus, unlike α-chymotrypsin, there is no evidence from these data of a dependence of the enzymic activity on a group ionizing in the pH range 8–10.

Inhibition by Tos-LysCH_2Cl. The inhibition of S.G. trypsin by a sevenfold molar excess of Tos-LysCH_2Cl at pH 7.0 was rapid and complete with activity dropping to zero in 20 min. The half-time for inactivation was 4 min. The presence of a 13-fold molar excess of the competitive inhibitor *p*-aminobenzamidine over Tos-LysCH_2Cl almost completely blocked the reaction with activity dropping only a few per cent in 20 min. These results confirm and extend the work of Yoshida *et al.* (1971) and indicate that the specificity and active sites of S.G. trypsin and bovine trypsin must be very similar. To further characterize the Tos-LysCH_2Cl-inhibited enzyme, amino acid analyses of the inhibited protein were performed and on comparison with the native enzyme (Jurášek *et al.*, 1969; Olafson and Smillie, 1975), it was shown to have 1.0 histidine residue per mole less than the native molecule. To demonstrate that the reagent had alkylated the single histidine at residue 57 of S.G. trypsin, a peptic digest of the inhibited enzyme was subjected to the cysteic acid diagonal techniques of Brown and Hartley (1966), previously employed for the isolation and sequence analyses of the disulfide bridged peptides of S.G. trypsin (Jurášek *et al.*, 1969). It was observed (see Figure 7A and B) that the Pauly-positive peptide P7-02 present in the digest of the native enzyme was absent in the inhibited enzyme and was replaced by a Pauly-negative peptide, P7-02*, migrating in the same position as peptide P7-04, the disulfide bridge mate of P7-02. These two peptides were separated by an additional electrophoresis at pH 1.8 and upon amino acid analysis gave the compositions reported in Table I. An additional ninhydrin positive spot (O-TLCK) was observed on the diagonal which upon amino acid analysis showed only a single component eluting in the position of tryptophan on the amino acid analyzer. This substance is believed to be a degradation product of the alkylated histidine arising from a peracid rearrangement during the performic acid oxidation step of the diagonal procedure. Such a rearrangement was postulated in the case of Tos-PheCH_2Cl[1]-inhibited chymotrypsin to yield 3-carboxymethylhistidine and a ninhydrin negative derivative (Stevenson and Smillie, 1968). In the case of Tos-LysCH_2Cl-inhibited S.G. trypsin this product is ninhydrin positive because of the presence of the primary amino group in the original alkylating reagent. The amino acid analyses reported in Table I identify peptides P7-02* and P7-04 as being derived from residues 54–64 and 39–46, respectively, of S.G. trypsin (Jurášek *et al.*, 1969; Olafson and Smillie, 1975). The recovery of one residue of 3-carboxymethylhistidine per mole of peptide P7-02* is fully consistent with the alkylation of histidine-57 during the inhibition of the enzyme by Tos-LysCH_2Cl. Thus, in this respect S.G. trypsin behaves identically with bovine trypsin.

Table I: Amino Acid Compositions of Cysteic Acid Peptides P7-02* and P7-04 Isolated from Tos-Lys CH_2Cl-Inhibited S.G. Trypsin.

Amino Acid	P7-02* (Molar Ratios)	P7-04 (Molar Ratios)
Cysteic acid	0.93	0.96
Aspartic acid	2.02	
Threonine	0.96	
Serine	1.89	0.99
Alanine	2.13	1.00
Glycine	2.10	3.06
3-Carboxymethylhistidine	0.98	
Valine	0.98	
Methionine sulfone		0.77
Leucine		1.00
NH_2 terminus	Threonine	Serine

Table II: Moffitt-Yang Parameters of S.G. Trypsin as a Function of pH.

pH	a_0 (deg)	b_0 (deg)
7.0	−48	−130
7.5	−67	−125
8.0	−63	−123
8.5	−59	−129
9.0	−84	−121
9.9	−163	−122

ORD and CD Studies. Measurements of the optical rotatory dispersion of S.G. trypsin were made in the visible region (300–550 nm) over a pH range of 7.0–9.9. The parameters a_0 and b_0 were calculated at each pH (Table II). There was no significant change in b_0 within this pH range although a_0 showed a sharp decrease between pH 9.0 and 9.9. ORD measurements were also performed in the ultraviolet range (200–250 nm) at several pH values from 7.3 to 10.1 (Figure 8) and CD measurements (190–250 nm) in the pH range 5.3–10.5 (Figure 9). These measurements gave no indication of a conformational change between pH 5.3 and 10.1 but did show a major change in the CD spectrum when the pH was elevated to 10.5 (Figure 9).

Acetylation and Carbamylation Studies. Attempts to modify the amino groups of S.G. trypsin by acetylation under the conditions described by Oppenheimer *et al.* (1966) led to extensive precipitation of the enzyme. Bz-ArgOEtase assays and NH_2-terminal analyses of the remaining soluble material indicated that although all the lysines had been modified, acetylation of the α-amino group was incomplete and a significant proportion (approximately

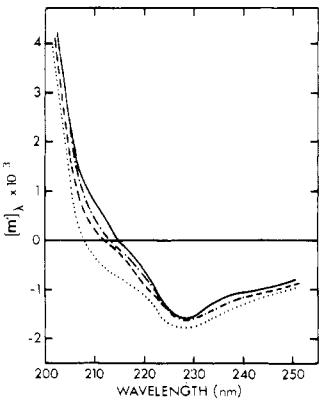

FIGURE 8: Optical rotatory dispersion of S.G. trypsin at pH 7.3 (—), pH 8.3 (- - -), pH 8.9 (- • -), and pH 10.1 (• • •).

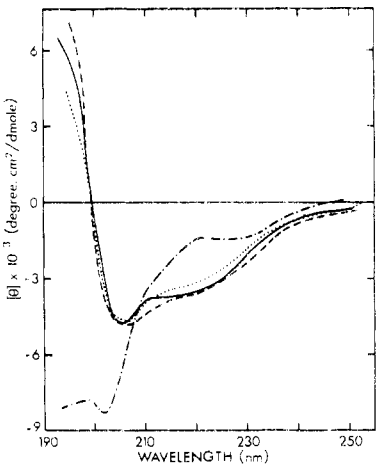

FIGURE 9: Circular dichroism of S.G. trypsin at pH 5.3 (- - -), pH 7.6 (—), pH 9.3 (• • •), and pH 10.5 (- • -).

30%) of the original activity of the enzyme remained. Similarily, reaction of trypsin with potassium cyanate under the conditions described in the Experimental Procedure produced a soluble product which retained a high proportion of its enzymic activity and whose α-amino group was incompletely carbamylated.

Discussion

The results described above indicate that S.G. trypsin has many of the same enzymic, chemical, and physical properties as bovine trypsin. Thus, in its high specificity toward arginyl and lysyl peptide bonds of the oxidized chains of insulin, its low K_m for the synthetic substrate *N*-α-benzoyl-L-arginine ethyl ester, its inhibition by reaction of its histidine-57 residue with Tos-LysCH_2Cl, and the effects of pH and Ca^{2+} on its esterase activity and stability are all properties shared by bovine trypsin. In addition the effects of replacing water by ^2H_2O on the pK_a of the ionizing group controlling the activity of the enzyme in the pH range 5.5–7.0 and on the rate of hydrolysis are similar to observations made with other serine proteases, indicating a catalytic mechanism for S.G. trypsin similar to these enzymes.

The effects of pH on the stability, activity, and ORD and CD properties of S.G. trypsin are of considerable interest in relation to the possible role of its terminal α-amino group in the stabilization of the architecture of the active site of the enzyme. For those mammalian serine proteases (α-chymotrypsin, elastase, and trypsin) whose tertiary structures have been elucidated by X-ray diffraction, the NH_2-terminal sequence is highly conserved and the charged α-amino group (either isoleucine or valine-16) shown to form a salt linkage with aspartic acid-194 in a hydrophobic environment (Blow *et al.*, 1969; Shotton and Watson, 1970; Stroud *et al.*, 1971). In the case of α-chymotrypsin several laboratories have observed that the state of ionization of the α-amino group appears to control the active conformation and substrate binding ability of the enzyme (Himoe *et al.*, 1967; McConn *et al.*, 1969). Deprotonation (Hess, 1971), acylation (Chevallier *et al.*, 1969; Oppenheimer *et al.*, 1966; Ghélis *et al.*, 1970) or deamination (Dixon and Hofmann, 1970) lead to its inactivation. However, other investigators have questioned the essentiality of a charged α-amino group in the case of δ-chymotrypsin. Valenzuela and Bender (1969, 1970) studied the δ-chymotrypsin-catalyzed hydrolysis of specific substrates and the binding properties of three competitive inhibitors over the pH range 7–11. Unlike α-chymotrypsin, δ-chymotrypsin was shown to be active and to bind substrates and inhibitors to a significant extent when isoleucine-16 was not protonated. Agarwal *et al.* (1971) observed that δ-chymotrypsin modified at the NH_2-terminal isoleucine-16 by reaction with either ethyl acetimidate or methyl picolinimidate retained its activity toward AcTyrOEt[1] at pH 8.0. In the case of elastase the importance of the protonated α-amino group has also been questioned. Although in the crystalline state the NH_2-terminal residue forms an internal ion pair with aspartic acid-194, Kaplan and Dugas (1969) appear to have shown that both native and fully acetylated elastase remain fully active at all pH values between 8 and 10. Other serine proteases also appear to differ from α-chymotrypsin in its requirement for a protonated NH_2-terminal residue. The α-lytic protease of Myxobacter 495, like elastase, shows no conformational change with pH between 5.0 and 10.5 and both the native and acetylated enzymes are fully active in the range of pH between 8.0 and 10.0 (Kaplan and Whitaker, 1969). Similarily the two *Streptomyces griseus* proteases A and B (or lysine-free chymoelastase and guanidine-stable chymoelastase) isolated from Pronase (Jurášek *et al.*, 1971) have recently been reported to have been acetylated without loss of enzymic activity (Siegal and Awad, 1973). In the case of bovine trypsin on the other hand, the integrity of the isoleucine-16 to aspartate-194 ion pair appears to be essential for its full activity. Robinson *et al.* (1973) have shown that chemical modification of the α-amino group of ϵ-guanidinated trypsin by carbamylation, thiocarbamylation, or amidination renders the enzyme inactive toward specific ester and amide substrates. These observations are consistent with the earlier observation that deamination of the α-amino group of trypsin by nitrous acid (Scrimger and Hofmann, 1967) causes inactivation of the enzyme. The present studies with S.G. trypsin are consistent with the interpretation that its α-amino group is buried and only becomes fully available for deprotonation and chemical modification upon denaturation and unfolding of the enzyme. Thus the activity of the enzyme shows no change between pH 8 and 10 and only decreases sharply when the enzyme becomes denatured at pH values above 10.1 as detected by the instability of the enzyme and by the change in the CD spectrum when the pH was increased to 10.5. The sharp decrease in the Moffitt parameter a_0 observed between pH 9.0 and 9.9 cannot be interpreted in terms of a conformational change in the absence of other evidence in view of the dependency of this pa-

rameter on environmental interactions with surface side chains as well as on internal side-chain interactions. The constancies of the esterase activity, of the b_0 parameter, and of the CD and ORD spectra between pH 9.0 and 10.1 argue against such an occurrence. Although the reactivity of the α-amino group of S.G. trypsin has not been investigated in a rigorous manner in the present work, our failure to effect its full acetylation or carbamylation under the conditions tested would be consistent with its relative unreactivity by virtue of its ion-pair formation with aspartic acid-194 in the native enzyme. In this respect then, also, S.G. trypsin appears to behave in an identical manner with bovine trypsin.

In summary, by the criteria tested, S.G. trypsin has almost identical enzymic, chemical, and physical properties as bovine trypsin. These remarkable similarities are shown in the accompanying paper (Olafson *et al.*, 1975) to be attributable to a high degree of homology in the amino acid sequences of the two enzymes and undoubtedly to their very similar tertiary structures.

Acknowledgments

The authors thank Dr. L. Jurášek for much helpful advice and discussion. We are indebted to Mr. M. Aarbo for the performance of the molecular weight determination and to Mr. K. Oikawa for performing the optical rotatory dispersion and circular dichroism measurements. The amino acid analyses were done by Mr. M. Nattriss.

References

Agarwal, S. P., Martin, C. J., Blair, T. T., and Marini, M. (1971), *Biochem. Biophys. Res. Commun. 43,* 510.

Bender, M. L., Pollock, E. J., and Neveau, M. C. (1962), *J. Amer. Chem. Soc. 84,* 595.

Bender, M. L., and Hamilton, S. A. (1962), *J. Amer. Chem. Soc. 84,* 2570.

Birk, Y. (1968), *Ann. N.Y. Acad. Sci. 146,* 394.

Blow, D. M., Birktoft, J. J., and Hartley, B. S. (1969), *Nature (London) 221,* 337.

Brown, J. R., and Hartley, B. S. (1966), *Biochem. J. 101,* 214.

Chevallier, J., Yon, J., and Labouesse, J. (1969), *Biochim. Biophys. Acta 181,* 73.

Coan, M. H., and Travis, J. (1970), *Comp. Biochem. Physiol. 32,* 127.

Dixon, J. W., and Hofmann, T. (1970), *Can. J. Biochem. 48,* 671.

Gates, B. J., and Travis, J. (1969), *Biochemistry 8,* 4483.

Ghélis, C., Garel, J., and Labouesse, J. (1970), *Biochemistry 9,* 3902.

Glasoe, P. K., and Long, F. A. (1960), *J. Phys. Chem. 64,* 188.

Green, N. M., and Neurath, H. (1954), *Proteins, 1st Ed., 2B,* 1057.

Gutfreund, H. (1955), *Discuss. Faraday Soc. 20,* 167.

Gutfreund, H., and Knowles, J. R. (1967), *in* Essays in Biochemistry, Vol. 3, Campbell, P. N., and Greville, G. D., Ed., New York, N.Y., Academic Press, p 46.

Hess, G. P. (1971), *Enzymes, 3rd Ed., 3,* 213.

Himoe, A., Parks, P. C., and Hess, G. P. (1967), *J. Biol. Chem. 242,* 919.

Hunkapiller, M. W., Smallcombe, S. H., Whitaker, D. R., and Richards, J. H. (1973), *Biochemistry 12,* 4732.

Jurášek, L., Fackre, D., and Smillie, L. B. (1969), *Biochem. Biophys. Res. Commun. 37,* 99.

Jurášek, L., Johnson, P., Olafson, R. W., and Smillie, L. B. (1971), *Can. J. Biochem. 49,* 1195.

Kafatos, F. C., Tartakoff, A. M., and Law, J. H. (1967), *J. Biol. Chem. 242,* 1477.

Kaplan, H., and Dugas, H. (1969), *Biochem. Biophys. Res. Commun. 34,* 681.

Kaplan, H., and Whitaker, D. R. (1969), *Can. J. Biochem. 47,* 305.

Kay, C. M., and Oikawa, K. (1966), *Biochemistry 5,* 213.

McConn, J., Fasman, G. D., and Hess, G. P. (1969), *J. Mol. Biol. 39,* 551.

Moffitt, W., and Yang, J. T. (1956), *Proc. Nat. Acad. Sci. U.S. 42,* 569.

Morihara, K., and Tsuzuki, H. (1968), *Arch. Biochem. Biophys. 126,* 971.

Oikawa, K., Kay, C. M., and McCubbin, W. D. (1968), *Biochim. Biophys. Acta 168,* 164.

Olafson, R. W., Jurášek, L., Carpenter, M. R., and Smillie, L. B. (1975), *Biochemistry,* following paper.

Oppenheimer, H. L., Labouesse, B., and Hess, G. P. (1966), *J. Biol. Chem. 241,* 2720.

Richards, E. G., Teller, D. C., and Schachman, H. K. (1968), *Biochemistry 7,* 1054.

Robinson, N. C., Neurath, H., and Walsh, K. A. (1973), *Biochemistry 12,* 420.

Scrimger, S., and Hofmann, T. (1967), *J. Biol. Chem. 242,* 2528.

Siegal, S., and Awad, W. M. (1973), *J. Biol. Chem. 218,* 3233.

Shaw, E. (1967), *Methods Enzymol. 11,* 677.

Shotton, D. M., and Watson, H. C. (1970), *Nature (London) 225,* 811.

Stevenson, K. J., and Smillie, L. B. (1968), *Can. J. Biochem. 46,* 1357.

Stroud, R. M., Kay, L. M., and Dickerson, R. E. (1971), *Cold Spring Harbor Symp. Quant. Biol. 36,* 125.

Travis, J., and Roberts, R. C. (1969), *Biochemistry 8,* 2884.

Trop, M., and Birk, Y. (1968), *Biochem. J. 109,* 475.

Valenzuela, P., and Bender, M. L. (1969), *Proc. Nat. Acad. Sci. U.S. 63,* 1214.

Valenzuela, P., and Bender, M. L. (1970), *Biochemistry 9,* 2440.

Wählby, S. (1968), *Biochim. Biophys. Acta 151,* 304.

Wählby, S., and Engström, L. (1968), *Biochim. Biophys. Acta 151,* 402.

Wählby, S., Zetterqvist, O., and Engström, L. (1965), *Acta Chem. Scand. 19,* 1247.

Walsh, K. A. (1970), *Methods Enzymol. 19,* 41.

Weber, K., and Osborne, M. (1969), *J. Biol. Chem. 244,* 4406.

Yoshida, N., Sasaki, N., and Inoue, H. (1971), *FEBS Lett. 15,* 129.