Purity and pH-Dependence of *Streptomyces griseus* Protease 1

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Pronase, the extracellular enzyme preparation from *Streptomyces griseus*, is a very complex mixture of various proteases.[1-3] Among these microbial enzymes are at least three serine proteases homologous to the mammalian pancreatic proteases.[4] The finding of homologous proteases from species of so widely differing phylogenetic origin was of considerable interest and prompted further investigations into the structure, mechanism and specificity of the microbial enzymes in relation to the pancreatic serine proteases. The complete primary structures of all the three Pronase serine enzymes have now been determined,[5-6] and the tertiary structure of one of these enzymes, SGP1 (identical with SGPB of Ref. 5), was recently published.[7] Studies on the mechanism and specificity of one of the other serine enzymes, SGP3 (identical with SGPA of Ref. 5), have also been reported (see for examples Refs. 8 – 10).

We have begun a kinetic investigation of the active site of SGP1, with a number of peptide substrates, with the ultimate goal of correlating some of the results from that study with the tertiary structure of the enzyme. As a prerequisite for the above-mentioned studies, we here present data on the purity and pH-dependence of a preparation of SGP1.

Purity. Although some data on the purity of SGP1 have been reported earlier,[11] we now provide further evidence for the purity, necessary prior to a detailed specificity study.

To remove inactive material from a preparation of SGP1 (a kind gift from Dr. L. B. Smillie), the enzyme was chromatographed on a 2.5 × 87 cm Sephadex G75 Superfine bed.[3] SGP1 was eluted with a K_{av} of 0.538, which is in good agreement with previously reported values of 0.557 and 0.560 for the "F-peak", which contains SGP1 to ~90 %.[3] Most of the SGP1 eluate was concentrated by ultrafiltration, the buffer was changed to 0.25 M NH_4HCO_3, pH 7.7, by chromatography on a Sephadex G-25 column, and the enzyme solution was frozen and lyophilized.

The purity of the enzyme was studied by polyacrylamide gel electrophoresis at pH 6.8 and 8.5 as described earlier.[2,12] Application of 20 μg of freshly chromatographed or lyophilized SGP1 to analytical gels (*cf.* Ref. 12) resulted in both cases in a single stainable band moving towards the cathode with exactly the same rate as band 1 (=SGP1) in Pronase. To obtain further evidence for the purity of the preparation, a sample of 100 μg of lyophilized SGP1 was run on a small preparative polyacrylamide gel.[2] The enzymatic activities associated with this preparation of SGP1 can be seen from Fig. 1. There is one main peak (fractions 28 – 31) which is active towards both casein and GPNA. These two activities appear to have the same relative intensities from fraction to fraction, thus providing strong evidence for the homogeneity of the peak. On the trailing edge of the peak are a few fractions (24 – 26) with faint activity towards casein and GPNA, constituting about 3 % of the total activity towards these substrates. These fractions, which show approximately the same relative activities towards casein/GPNA as fractions 28 – 31, may either simply be due to "tailing" of the main peak or, alternatively, may constitute a modified form of SGP1 (corresponding to band 2 of Ref. 2) with a slightly different charge, but with essentially the same relative activities towards casein/GPNA as SGP1. Such microheterogeneities have been found to be formed during certain preparation methods for the closely related enzyme SGP3.[13] No activity towards L-Leu-β-naphthylamide could be demonstrated in any of the 50 fractions, which indicates that the preparation is free from the aminopeptidases present in Pronase.[2,3] A very small activity towards BANA was found in fractions 17 – 19, which corresponds well with the

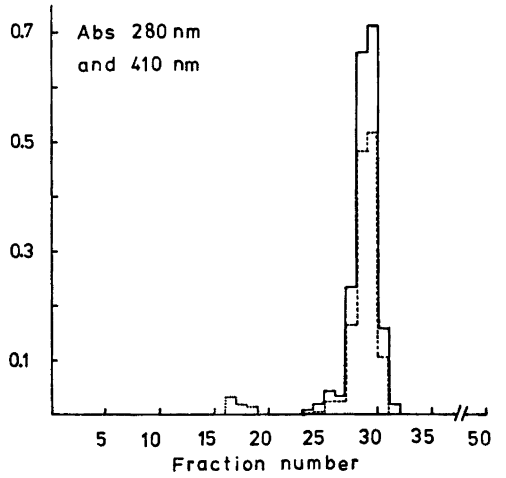

Fig. 1. Histogram showing enzymatic activities of a preparation of SGP1, found in the different gel fractions of a small preparative polyacrylamide gel after 3 1/3 h electrophoresis (cathodic migration) in 0.38 M borate – 0.01 M Ca^{2+} buffer, pH 6.8. The gel fractions (1.5 mm thick) were mashed and extracted with a 0.03 M borate buffer pH 7.5, 0.03 M in $CaCl_2$. Activity towards casein (–), glutaryl-L-Phe-*p*-nitroanilide = GPNA (- - -), and benzoyl-DL-Arg-*p*-nitroanilide = BANA (· · ·) at pH 7.5.

position of SGP4 [2] or *Streptomyces griseus* trypsin.[6] By comparing the activity of fractions 17–19 with that of a known amount of SGP4, it has been possible to estimate the amount of enzyme present in the fractions to be less than 0.1 % of the total amount of enzyme (100 μg) applied to the gel. Since the BANA activity is completely separated from the SGP1 fractions 28–31, it is clear that there is no intrinsic "trypsin-like" activity in SGP1.

In conclusion, the results provide evidence that this preparation of SGP1 is at least 99.9 % pure with respect to its activity towards casein/GPNA. A small amount (about 3 %) of this activity was found in a peak closely associated with the main (97 %) peak (=SGP1) of the preparation. However, because the relative activities towards casein/GPNA of the small peak are similar to those of the main peak, this would seem unlikely to affect significantly the kinetic properties of the preparation.

pH-Dependence. Besides providing information about the pK_a of catalytically important residues, knowledge of the pH-dependence of an enzyme makes it possible to chose optimal conditions for kinetic studies. The pH-dependence of k_{cat} for the SGP1-catalyzed hydrolysis of the uncharged substrate Ac-Pro-Leu-*p*-nitroanilide [14] in the pH-interval 4.1–10.8 can be seen from Fig. 2. k_{cat} increases with increasing pH up to about pH 8, being constant from pH 8 up to at least pH 10.8. K_m, on the other hand, is virtually constant ($K_m = 3 \pm 1$ mM) within the investigated interval, and the pH-dependence of SGP1 therefore shows close similarity with those of SGP3,[15] α-lytic protease [16] and elastase,[17] but differs from that of chymotrypsin,[18] where K_m increases quite rapidly above pH 8. Fig. 2 suggests that the pH-dependence of SGP1 is dependent on an ionizing group with an apparent pK_a of about 6.7. This value is almost identical with the pK_a value (6.6) previously determined for the pH-dependence of SGP3,[15] and is very similar to the pK_a's of other homologous serine proteases.[16,18] In α-lytic protease [19] and trypsin,[20] there is now evidence that the group responsible for this pK_a is Asp-102 and not, as earlier believed, His-57.[16,18] It might be expected that Asp-102 is responsible for the pK_a around 6.7 of the pH-dependencies of other homologous serine proteases as well, including SGP1 and SGP3.

Gertler has reported $pK_{a1} = 6.6$ and $pK_{a2} = 9.5$ for the pH-dependence of the second order rate constant of inhibition of SGP1 by Ac-Leu-Phe chloromethyl ketone.[21] While $pK_{a1} = 6.6$ is in excellent agreement with the value obtained in the present investigation, Fig. 2 does not provide any support for a pK_{a2} around 9.5, but indicates that a pK_{a2} value must be greater than 10.8. A possible explanation for the low pK_{a2} of 9.5 might be that chloromethyl ketones are unstable in water solutions of higher pH's, and will decompose quite rapidly in alkaline media.[22,23] Consequently, the concentration of chloromethyl ketone in an inhibition mixture at alkaline pH will decrease more or less rapidly depending on the pH. The result will be an apparent slower inhibition of SGP1 at higher pH's, and this effect might well account for the low pK_{a2} of 9.5.

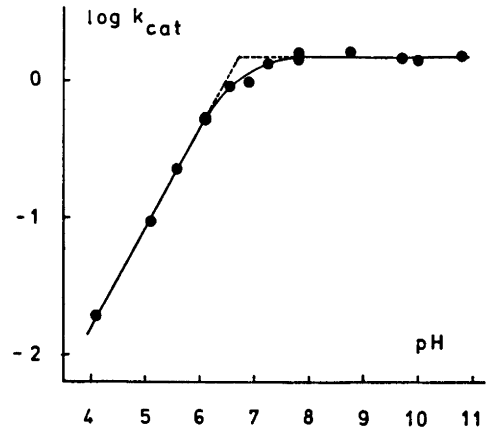

Fig. 2. pH-Dependence of k_{cat} for the SGP1-catalyzed hydrolysis of Ac-Pro-Leu-*p*-nitroanilide at 37 °C followed at 410 nm. The reaction-solutions contained 0.2–1 mM substrate in 1.1 ml 50 mM buffer (acetate, maleate, tris or glycine-buffer), 10 mM $CaCl_2$ with 2.5 % acetone and 0.17–3 μM enzyme. Enzyme-free blanks, otherwise identical in composition to the reaction mixtures, were used throughout the measurements.

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Formation of α-Allenic Dithioketals in a [2,3] Sigmatropic Rearrangement of Intermediates Derived from *S*-Propargylic Dithioesters*

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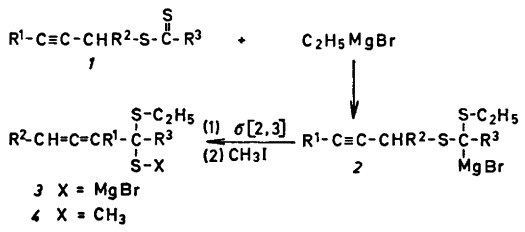
Alkyl Grignard reagents may react with thiocarbonyl compounds, such as thioketones and dithioesters, under thiophilic addition.[1] Recently it was reported [2] that thiophilic addition of Grignard reagents to *S*-allylic dithioesters is followed by a [2,3] sigmatropic shift of the intermediate carbanion, which gives rise to dithioketals of β-unsaturated ketones. Closely related [2,3] sigmatropic rearrangements of α-metallated allylic thioethers have also been observed.[3]

This communication reports that the thiophilic addition of ethylmagnesium bromide to the double bond of the thiocarbonyl group of *S*-propargylic dithioesters in THF at −30 °C is spontaneously followed by a [2,3] sigmatropic rearrangement of the intermediate carbanion. Subsequent methylation of the so formed allenic thiolate gives α-allenic dithioketals in 58 − 74 % yield, (see Scheme 1). These dithioketals represent a hitherto unknown type of allenic compound.

Scheme 1. α-Allenic dithioketals *4a − d* from the reaction of *S*-propargylic dithioesters *1a − e* with ethylmagnesium bromide.



Compound	R^1	R^2	R^3	Yield [a] of *4* (%)
1a [5b]	H	H	C_2H_5	64
1b [7]	CH_3	H	C_2H_5	69
1c	C_6H_5	H	C_2H_5	58
1d	CH_3	H	C_6H_{13}	74
1e	CH_3	CH_3	C_2H_5	–

[a] Isolated by column chromatography.

The allene-forming [2,3] sigmatropic rearrangement in a propargylic system described here is closely related to several analogous reactions known from the literature, especially those in propargylic sulfonium ylides,[4] which afford allenic sulfides.

The propargylic dithioesters *1a − e* were prepared according to the literature.[5] A mixture of THF and HMPT was used as the solvent for the alkylation of the alkyldithiomagnesium bromide with the appropriate propargylic bromide. The dithioesters were heat sensitive and could not be distilled at 10 Pa without decomposition but had to be purified by column chromatography.

The thiophilic addition of ethylmagnesium bromide to the thiocarbonyl group of the *S*-propargylic dithioesters *1a − e* is a smooth reaction at −30 °C. THF is used as the solvent,

* Allenes and Acetylenes XV. Part XIV: *Acta Chem. Scand. B 30* (1976) 521.