Subtilopeptidase A Isoenzyme System

INTERACTION WITH SERUM COMPONENTS AND ITS IMPORTANCE FOR QUANTITATIVE IMMUNOELECTROPHORESIS

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A method was developed which involved electroimmunoassay and crossed immuno-electrophoresis of subtilopeptidase A (EC 3.4.21.14). Initial trials with unfractionated antiserum were not successful and interaction of the enzyme with non-immunoglobulin serum components were shown to be the cause of the failures. Quantitative immuno-electrophoresis was possible when purified immunoglobulins were used. A pH of 6.5 (lower than the usual pH 8.6) was necessary to obtain a proper baseline definition. Subtilopeptidase A was confirmed as a multiple isoenzyme system. Qualitative inter-batch variations were detected. Di-isopropyl phosphorofluoridate inhibition altered the electrophoretic pattern, but no loss of antigenic determinants was observed.

Earlier work (Zuidweg *et al.*, 1972; Verbruggen *et al.*, 1974; Verbruggen & Baeck, 1975) demonstrated considerable electrophoretic heterogeneity in commercial crystalline subtilopeptidase A. Autodigestion and Ca^{2+} -binding effects could be excluded as the cause of this heterogeneity. Commercial enzyme preparations were also shown to be immunochemically heterogeneous (Verbruggen *et al.*, 1974; Belin *et al.*, 1970).

These findings prompted me to investigate the feasibility of quantitative immuno-electrophoretic analysis with the method developed by Laurell (Ganrot, 1972) and Freeman (Weeke, 1973), and reviewed extensively by the present author (Verbruggen, 1975).

Since subtilopeptidase is a basic protein with a low charge, we first developed a technique for improved and faster agarose-gel electrophoresis of the isoenzyme system in veronal buffer at pH 6.5 (Verbruggen & Baeck, 1975). This technique was used to achieve one-dimensional and two-dimensional quantitative immuno-electrophoresis. It made the whole antigen system cathodal.

Materials and Methods

Electrophoresis and immuno-electrophoresis

Agarose-gel electrophoresis was described by Johansson (1972). A veronal buffer system, pH 6.5, was used in two dimensions (Verbruggen & Baeck, 1975). In later work it was used for the first-dimension

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runs only. In the second dimension, no resolution is required. Therefore the second dimensions could be run in 0.02M-Tris-phosphate or sodium phosphate buffer at pH 6.5 or 6.0 respectively. The pH 7.1 veronal buffer preparation was also described by Verbruggen & Baeck (1975). Other buffers were prepared in the usual way. 'A-37' agarose was purchased from I.B.F. (Gennevilliers, Seine, France) and deionized (Johansson & Stenflo, 1971).

The general procedures used for crossed immuno-electrophoresis and electroimmunoassay were as published (Weeke, 1973; Laurell, 1972; Ganrot, 1972). Tandem immuno-electrophoresis (Krøll, 1973) was done in our veronal buffer, pH 6.5, system. Gels of 1 mm thickness were used. The power supply was set at constant current and adjusted to 10–20 V/cm for the first dimension and 3–5 V/cm for the second dimension. Results were photographed with the primary cathode at the right hand, and the secondary cathode at the bottom.

Enzyme

The enzyme preparations examined were Carlsberg subtilisins, either industrial product [supplier's assay 4.5 A.U. (Anson Units)] or crystalline material (supplier's assay 24–32 A.U.), from Novo Industri (Copenhagen, Denmark). This enzyme is more accurately designated subtilopeptidase A (EC 3.4.21.14); for brevity it is also referred to as 'subtilisin' or 'the enzyme' in this report. The batch numbers are given in the Plate legends.

Enzyme activity and inhibitors. Proteolytic activity and its inhibition by serum components were conveniently assayed by visual observation of tubes containing black photographic paper and enzyme in the

presence and absence respectively of serum. The enzyme attacks the gelatin on the strip of black photographic paper which leads to the formation of a dark turbid gelatin suspension in the tube. Such assays yield qualitative and semi-quantitative results. To normalize these tests, all tubes were provided with the same liquid volume and the same total mass of protein. This was done by adding an amount of dissolved gelatin equivalent to the serum to the tubes without serum.

Inhibition of enzyme activity by serum components was detected by first doing a regular agarose-gel electrophoresis of human serum. After the run the gel slab containing the separated bands was soaked in enzyme solution for 45 min (0.4 mg of subtilisin/ml of 0.075 M-veronal buffer, pH 8.6). The enzyme diffused into the gel slab and after a thorough rinse with tap water, black photographic paper was applied to the gel for about 15 min. Enzyme taken up by the gel slab attacked the gelatin except at those places where it had been inhibited by a serum band.

To relate molecular weight of the inhibitors to their electrophoretic mobility, a fresh agarose-gel electrophoresis slab was prepared containing the bands from a run of a human serum sample containing 5% of the enzyme. Another gel slab homogeneously mixed with whole antiserum against the enzyme was prepared and laid over the electrophoresis bands. Counterdiffusion was allowed to take place overnight. The slabs were then separated and soaked for 48 h in 0.9% NaCl (five changes) to remove unprecipitated protein. Precipitates in both slabs were then stained with Coomassie Brilliant Blue (Uriel, 1966).

Antiserum and antibodies

Antiserum against subtilisin was obtained by conventional immunization of rabbits with an emulsion of an industrial enzyme preparation in Freund's complete adjuvant. Serum was collected from several animals by repeated bleedings and pooled. It was used as such or fractionated. Fractionation included, in the following order: treatment with octanoic acid (Steinbuch *et al.*, 1970), twofold precipitation with 1.8 M-(NH₄)₂SO₄ and chromatography on a column (2.5 cm × 40 cm) of DEAE-cellulose (DE-52; Whatman, H. Reeve Angel and Co., London E.C.4, U.K.) to remove trace impurities left. Rabbit antibody against whole human serum was purchased from Dakopatts, Copenhagen, Denmark.

Autodigestion

The possible effects of autodigestion on electroimmunoassay of the enzyme were examined by using unfractionated antiserum as follows. An enzyme solution was incubated in 0.075 M-veronal buffer, pH 8.6, at 55°C. Portions were withdrawn at times

0, 1, 3 and 6 h and frozen immediately. Samples from these portions were run simultaneously in one electroimmunoassay plate.

Inhibition

Di-isopropyl phosphorofluoridate inhibition of subtilopeptidase was studied by stirring the enzyme, dissolved in 0.1 M-calcium acetate buffer, pH 6.5, overnight with at least a 30-fold molar excess of the inhibitor solution in propan-2-ol (1 ml of di-isopropyl phosphorofluoridate + 19 ml of propan-2-ol). Di-isopropyl phosphorofluoridate was purchased from Aldrich, Milwaukee, Wis., U.S.A. Excess of inhibitor was removed by four to five precipitations with saturated (NH₄)₂SO₄. The final precipitate of protein and CaSO₄ was extracted with distilled water. The extracts containing inhibited enzyme were freeze-dried.

Results

Irregularities in electroimmunoassay

Our first attempts to perform electroimmunoassay were with whole antiserum in 0.075 M-veronal buffer at pH 8.6 or 8.2. Even at this high pH, most of the protein migrates toward the cathode during agarose-gel electrophoresis. However, in the immunogel for the electroimmunoassay, subtilisin moved predominantly towards the anode. The precipitates formed were ill-defined and non-quantifiable (Plate 1a). Dialysis of the antiserum did not improve or alter the results. The possibility that the poor results were due to autodigestion of the proteinase was examined as described in the Materials and Methods section. The resulting precipitates clearly show that the results for autodigested enzyme are no worse than those for non-digested material (see Plate 1b). Thus autodigestion is not the cause of the retrograde enzyme mobility. Even the non-digested enzyme behaves in an irregular fashion. During the many experiments of this kind, the results were largely unpredictable and variable.

Enzyme inhibition by serum

Tube tests for proteolytic activity showed that rabbit serum, like human serum, greatly decreases the proteolytic activity, whereas an equivalent protein mass of gelatin did not measurably depress the activity. On testing the fractions of human serum [obtained by gel filtration on Sephadex G-200 (Pharmacia, Uppsala, Sweden)] in the same way, inhibition was found in the first and the last peaks.

The inhibitory activity was located in the electrophoretic pattern of human serum as described in the Materials and Methods section. The α_1 and α_2 zones showed inhibition (Plate 2a). The α_2 inhibition area

EXPLANATION OF PLATE I

Electroimmunoassay attempted with unfractionated antiserum

(a) Electroimmunoassay of crystalline subtilisin Carlsberg (lot no. 60420; 24.4 A.U.) with unfractionated antiserum from rabbit in 0.02M-phosphate buffer, pH 7.0. The run was for 16h. Cathode downwards. From left to right, the following quantities of dry enzyme were applied (μ g): 1; 2; 3; 4; 5; 6. (b) Electroimmunoassay of autodigesting subtilisin Carlsberg (lot no. 60420; 24.4 A.U.) with unfractionated antiserum from rabbit. The buffer was 0.076M-veronal buffer, pH 8.6. Autodigestion was at 55°C over (left to right) 6, 1, 0, 1, 3, 6 or 0h. The quantity was equivalent to 2.5 μ g of dry undigested enzyme per well. The run was for 16h. Cathode downwards.

EXPLANATION OF PLATE 2

Interaction of subtilisin with serum components

(a) Demonstration of enzyme inhibition by human serum. This was done as described in the Materials and Methods section. Anode upwards. The run was for 1.5 h at about 10 V/cm in 0.05 M-veronal buffer, pH 8.2, with (left to right) 8, 6 and 4 μ l of serum. Note the two dark zones, the α_1 and α_2 regions, the upper one being very diffuse. (b) Incidental demonstration of the binding of subtilisins by serum components. Regular agarose-gel electrophoresis of the enzyme was run. In a previous experiment on the same apparatus, an electroimmunoassay had been run with unfractionated antiserum. This resulted in a distorted electrophoresis pattern, as explained in the text. (c) Assignment of molecular-weight ranking to inhibitors in serum, as described in the Materials and Methods section. Electrophoresis gel to the left; antiserum gel to the right. The pre-albumin precipitate was almost invisible in the gels; the very faint α_1 precipitate is stronger in the antiserum gel; the α_2 precipitate dominates in the electrophoresis gel. Anode upwards. Electrophoresis was in 0.05 M-veronal buffer, pH 8.2, for 1 h at 10 V/cm. (d) Combined patterns for agarose-gel electrophoresis and crossed immunoelectrophoresis of (A) human serum and (B) a solution of subtilopeptidase in serum. The buffer was 0.05 M-veronal, pH 8.2. The first dimension was run for 1.5 h at 13 V/cm. The second dimension was run for 22 h at 3.5 V/cm. The immunogels contained 1.25 μ l of anti-(whole-human-serum) immunoglobulin (Dakopatts)/cm². Anodes are to the left (electrophoresis) and upwards (immunoelectrophoresis). Samples: run (A) 1 μ l of human serum in the agarose-gel electrophoresis; 2 μ l of human serum in the two-dimensional run; run (B) 1.33 μ l of human serum containing 13.3 μ g of crystalline subtilisin (batch no. 90919) in the agarose-gel electrophoresis; 2 μ l of serum containing 20 μ g of enzyme in the two-dimensional run. Note the changes due to the enzyme, mainly the peak with pre-albumin mobility, showing partial identity with the decreased albumin peak. Free enzyme bands are visible cathodally from the application slit in the first dimension of run (B).

EXPLANATION OF PLATE 3

Electroimmunoassay of crystalline subtilopeptidase A versus purified rabbit antibody

(a) In veronal buffer (pH 6.5) left to right 0.5, 1, 2, 3, 4 and 5 µg of dry enzyme (batch no. 90708)/well. They were run overnight at 5 V/cm. Cathode at the bottom. Only one antigenic component was found although some double contours can be discerned, as explained in the text. (b) In 0.02M-sodium phosphate buffer, pH 6.0; peak size for 1 µg of dry enzyme is indicated by the arrow (batch no. 80115). It was run overnight at 4 V/cm with the cathode downwards. Darker but faint small 'rockets' inside the main precipitates reveal the minor component as explained in the text. (c) Electroimmunoassay for the minor component in crystalline subtilopeptidase A (batch no. 90919) in veronal buffer (pH 7.1). Note the linearity as well as the fact that part of the precipitate is still anodal. The major component goes out of scale. It was run 20 h at 4 V/cm with the cathode downwards. The quantity applied was consecutively from 10 µg down in 1 µg steps to 1 µg/well. (d) Agarose-gel electrophoresis of crystalline subtilopeptidase A compared with the purified antibodies, against the enzyme. From left to right: di-isopropyl phosphorofluoridate-inhibited enzyme from batch no. 80115; active enzyme from batch no. 70-3; di-isopropyl phosphorofluoridate-inhibited enzyme from batch no. 70-3; immunoglobulin anti-subtilisin.

EXPLANATION OF PLATE 4

Combined patterns for agarose-gel electrophoresis and crossed immunoelectrophoresis of crystalline subtilopectidase A

Enzyme from batch no. 90919 was in position 2 (gel B) for simple electrophoresis and in gel C for crossed immunoelectrophoresis. Gel pattern A and position 1 (gel B) are enzyme from batch no. 70-3 (no minor component). In enzyme from batch no. 90919 an additional band, not seen in enzyme from lot no. 70-3 is visible. The gels were run in veronal buffer, pH 6.5, with the primary cathode downwards and the secondary cathode as indicated.

EXPLANATION OF PLATE 5

Crossed immunoelectrophoresis of inhibited subtilopectidase A

(a) Combined patterns for agarose-gel electrophoresis and crossed immunoelectrophoresis of crystalline subtilopectidase A after inhibition with di-isopropyl phosphorofluoridate. Cathode to the right and downwards; first dimension 3 h at 12 V/cm; second dimension 20 h at 4 V/cm in veronal buffer, pH 6.5. The enzyme was run into purified anti-enzyme immunoglobulins. Pattern A is the non-inhibited enzyme from the same batch (batch no. 80115) for comparison with the inhibited enzyme (patterns B and C). Note the bimodal main peak in the crossed pattern (C). (b) Tandem crossed immunoelectrophoresis on crystalline subtilopectidase A with 15 μ g of active enzyme (batch no. 70-3) in the right-hand (most cathodal) well and 15 μ g of di-isopropyl phosphorofluoridate-inhibited enzyme (batch no. 8011) in the left-hand well. The conditions are the same as for Plate 4.

EXPLANATION OF PLATE 6

Crossed immunoelectrophoresis of industrial subtilopeptidase A preparations

(a) and (b), 15 μ g of industrial enzyme was used and the conditions were as in Plate 5 but for the second dimension. The minor population components (ω) cannot be clearly discerned in the reproductions. Run (a), second dimension buffer was 0.02 M-sodium phosphate, pH 6.0; run (b), second dimension was 0.02 M-Tris-phosphate, pH 6.5. (c) and (d), 800 μ g of industrial enzyme was used. (c) Real run of enzyme in veronal buffer, pH 7.1 (Verbruggen & Baeck, 1975). Other conditions were as for Plate 4. In this preparation four major isoenzymes (ψ) and three to four minor ones (ω) can be seen as well as a faint trace component. (d) Diagrammatic pattern of (c), indicating the new nomenclature proposed, with the former designations in parentheses.

was better localized and more distinct than the α_1 area. Thus human serum and rabbit serum both seem to inhibit and complex subtilopeptidase A. Further evidence for complexing of subtilisin by serum components was obtained by accident but is relevant. An electrophoresis plate with samples of the enzyme was run (Verbruggen *et al.*, 1974), but just before this regular electrophoresis run of a cathodal enzyme, we had attempted to do an electroimmunoassay of the enzyme with unfractionated antiserum using the same apparatus. This had failed as described under 'Irregularities in electroimmunoassay'. For the electroimmunoassay a plate of 10cm width was used, whereas the electrophoresis plate was 20cm wide. In the electrophoresis apparatus used the contact between electrophoresis plate and electrolyte vessels was made with blocks of agarose (used for many runs without replacement). To save buffer, the electrode polarity was reversed between the electroimmunoassay and the electrophoresis run. The resulting electrophoresis pattern of the enzyme was grossly deformed. Normal enzyme that was expected to run towards the cathode, moved towards the anode in that section of the apparatus where the electroimmunoassay plate had been just before. Plate 2(b) shows enzyme of normal behaviour on the far right side. However, in the middle of the plate, which was where the electroimmunoassay plate had been, the bands have moved anodally from the application slit. The multiband pattern of the normal enzyme (right side of the plate) is also seen in the anodal protein (middle section of the plate).

Overlaying the serum-enzyme electrophoresis slab with a gel containing anti-subtilisin antibody resulted in three precipitates (Plate 2c). Each is visible in both slabs; a first faint one is in the pre-albumin zone, a second one in the slow α_1 zone and a third prominent one in the α_2 zone. The third one was more pronounced in the original electrophoresis slab whereas the second one came out darker in the antibody-containing gel. The first was too faint to make such a judgement.

Enzyme-serum interactions

To obtain more details of the interactions between human serum and subtilisin, normal agarose-gel electrophoresis and crossed immunoelectrophoresis against anti-(whole human serum) antibody were used. The pattern of a sample of normal human serum was compared with the one of the same serum made 5% with respect to the enzyme preparation and kept for a short time at 0°C. Drastic changes are revealed on the addition of enzyme (Plate 2d).

Electroimmunoassay

By using the purified immunoglobulin preparation against subtilisin, electroimmunoassay yielded well-

defined precipitation 'rockets' in the special pH6.5 veronal-buffer-electrophoresis system (Plate 3a) as well as in a regular electroimmunoassay system with a 0.02M-sodium phosphate buffer at pH6.0 (Plate 3b). At higher pH, in veronal buffer at pH7.1 (regular apparatus), a minor portion of the antigen is still anodal (Plate 3c). Therefore the buffer pH had to be lowered further to 6.5.

To check whether the isolated immunoglobulins are sufficiently immobile in veronal buffer, pH6.5, when compared with the antigen to be examined, antibody and antigen were run side by side on an agarose-gel electrophoresis plate (Plate 3d).

Crossed immunoelectrophoresis

In crossed immunoelectrophoresis with the same antibody preparation in the veronal buffer system at pH6.5, the major bands from the electrophoretic pattern yielded fused precipitation peaks. Some minor fused peaks, antigenically not identical with the major peaks, were found at the cathodal edge. Two selected batches yielded different crossed immunoelectrophoretograms, clearly corresponding to different first-dimension electrophoresis patterns (Plate 4).

Effect of di-isopropyl phosphorofluoridate inhibition

Since it was found in earlier work (Verbruggen & Baeck, 1975) that the electrophoresis pattern at pH6.5 of subtilisin changed on inhibition by di-isopropyl phosphorofluoridate, the inhibited enzyme was also examined in crossed immunoelectrophoresis as shown in Plate 5(a). Tandem crossed immunoelectrophoresis (Krøll, 1973) was run with the native and the inhibited enzyme (Plate 5b).

Industrial raw materials

In Plates 6(a) and 6(b) crossed immunoelectrophoresis of industrial preparations of subtilisin are shown. The second dimension was run in phosphate, with either sodium (pH6.0) or Tris (pH6.5) as counterion. Plates 6(c) and 6(d) show real and diagrammatic crossed immunoelectrophoretic patterns for another sample of industrial enzyme as run in veronal buffer, pH7.1, in both dimensions.

Discussion

Quantitative immunoelectrophoresis requirements

In crossed immunoelectrophoresis, antigens are first subjected to regular agarose-gel electrophoresis. Afterwards, the antigens are forced electrophoretically into an antibody-containing gel at right angles to the original separation (Ganrot, 1972; Weeke, 1973). In electroimmunoassay the first step is omitted

(Laurell, 1972). Therefore successful work requires a good agarose-gel electrophoretic separation in the first dimension. This was achieved in veronal buffer at pH 6.5 (Verbruggen & Baeck, 1975).

In addition, straight electroimmunoassay must be feasible and therefore stationary antibodies are required. Consequently we checked whether the immunoglobulin preparation used was sufficiently immobile during electrophoresis, compared with the mobility of the enzyme to be studied. Therefore we ran enzyme and antibodies simultaneously in agarose electrophoresis using the pH 6.5 veronal buffer system (Plate 3*d*). The large mobility difference is sufficient to make crossed immunoelectrophoresis feasible under the conditions used, eliminating to a certain extent the need for modified antibodies (Bjerrum *et al.*, 1973), as can be seen from the results (Plates 3*a*, 3*b*, 4, 5*a*, 5*b*).

Irregular immunoprecipitation

Plate 1(*b*) shows that autodigestion cannot explain why the cathodal mobility in the first dimension is not similarly obtained in the second dimension, nor why we achieve such poor cone definition. In fact, the non-incubated samples in Plate 1(*b*) give the worst results. Similar experiments gave largely unpredictable and variable results. As other enzymes did not show this strange behaviour, we concluded that a specific interaction must have occurred between an antiserum component and the antigens studied. A similar observation of perturbed mobility in the second dimension, in work on cathodal honey-bee mid-gut endopeptidases, was reported but left unexplained (Giebel *et al.*, 1971). As dialysis of the antiserum did not affect this mobility inversion, we concluded, after careful controls on all physical parameters, that the interference must be due to a macromolecular constituent in the serum. The hypothesis that this specific macromolecular factor was a proteinase inhibitor, by analogy with the trypsin inhibitors in serum, was both logically and operationally attractive.

Serum inhibitors

The tube tests proved the occurrence of inhibition by serum, and the gel-filtration work demonstrated the presence of at least two inhibitors. The lack of inhibition by the addition of equivalent protein amounts, in the form of gelatin, proved that the inhibition was not due to a preference for dissolved protein as a substrate rather than for the gelatin of the photographic plate.

The localization of the inhibitory capacity of the serum in the α_1 and α_2 areas suggested even more an analogy with the known serum trypsin inhibitors. Therefore we tried to correlate the α_1 inhibitor with a low-molecular-weight inhibitor and the α_2 with a

high-molecular-weight inhibitor by the overlaying experiment, which is somewhat analogous to immunofixation (Alper & Johnson, 1973). From the results, we deduced on the basis of diffusion velocities that the α_2 inhibitor has a higher molecular weight than immunoglobulin G, but the reverse was true for the α_1 inhibitor. Only on the basis of crossed immunoelectrophoresis could conclusions be drawn about the fastest-moving but faint precipitate complex found in this experiment.

Interactions with serum components

On addition of enzyme to serum, the patterns obtained from both regular agarose-gel electrophoresis and crossed immunoelectrophoresis immediately undergo drastic alterations (Plate 2*d*). Such mixtures are very complex. The high proteolytic activity added, as well as the limited scope of the work performed in that direction, make valid deductions difficult. There is considerable alteration of the serum, probably most of it caused by proteolytic attack, which allows for a multitude of interactions. One conspicuous feature of the enzyme action is the decrease in the size of the main peak of the serum pattern, i.e. albumin, with a concomitant appearance of a faster-moving species, which is antigenically partially identical with albumin (Bock & Axelsen, 1973). This was interpreted as a split of the serum albumin caused by subtilisin, producing a faster-moving fragment of smaller size. The finding of a species with pre-albumin mobility that reacts with anti-subtilisin antibody in the overlaying experiment suggests that this fast-moving subtilisin ligand is the same split product of albumin. This was not investigated further. These findings can be related to work done on the fragmentation of albumin by subtilopeptidase B by Adkins & Foster (1966), although their conditions were quite different. These authors split serum albumin into four fragments without characterizing them immunologically. Our finding is reminiscent also of the action of subtilopeptidase A on ovalbumin, as described by Linderstrøm-Lang & Ottesen (1947).

The product of the serum albumin split, demonstrated here as a weaker precipitate, seems to be a well-defined entity with a decreased number of antigenic determinants. It might therefore be of considerable importance in work on the antigenic and surface structure of human serum albumin. In sequencing work, too, it might be useful. Further, the binding phenomenon may shed light on the binding properties of whole albumin.

The observations on Plate 2(*b*) were interpreted as follows: inhibitors present in the whole antiserum from the electroimmunoassay were trapped in the agarose-gel contact blocks. On polarity reversal these molecules of fast mobility were swept in the reverse direction across the middle section of the

enzyme electrophoresis plate, and they formed complexes with the enzyme to give particles of opposite electrical charge. Free serum components, being considerably faster in movement than the enzyme, moved out of the plate. The reversed mobility of complexed enzyme in the middle section of the electrophoresis plate is analogous to the irregularities observed in attempts to run electroimmunoassay with unfractionated antiserum. This analogy explains why these electroimmunoassay attempts failed with antisera containing complexing components.

The fact that the original band pattern of the native enzyme is preserved after binding to serum ligands is strong evidence that the individual enzyme bands each consist of species with intact binding sites for the serum ligands.

At this stage of the work, the findings of Dolovich and his co-workers about interaction of the serum trypsin inhibitors with subtilisin were published (Dolovich *et al.*, 1971*a,b*; Dolovich & Wicher, 1971). Another report from the same authors indicated first that albumin does not bind radioactively iodinated (possibly altered) enzyme (Wicher & Dolovich, 1971). Later work, however, brought positive evidence for subtilisin-albumin complex-formation (Wicher & Dolovich, 1972). Very early but vague reports on the inhibition of subtilisin by serum were published by Ramon (1944) and by Richou (1960).

Although the interaction with serum components was initially found by using rabbit serum in our laboratory, all further research was done on human serum. Extrapolating to the rabbit, we considered it necessary to use highly purified immunoglobulin to achieve proper crossed immunoelectrophoretograms. Partial removal of ballast protein would risk leaving potentially interacting species in the antibody preparation, such as plasminogen, complement factors and most importantly traces of albumin. Therefore the rather extensive purification procedures described above were used.

Electroimmunoassay

With the purified immunoglobulin preparation, electroimmunoassay was possible at pH 6.5 and 6.0 (Plates 3*a* and 3*b*). Plate 3(*d*) demonstrates that unmodified immunoglobulins used as precipitins are sufficiently immobile in comparison with the antigens in our veronal buffer, pH 6.5, to make the technique feasible. The feasibility of electroimmunoassay in buffers with pH values below 8 has been reported by others. However, no work at pH values lower than 7.4 has before been published, except when modified antibodies were used, or in reverse systems (antibody precipitating in antigen-containing gels) (Verbruggen, 1975). The assay could distinguish between two antigenically different components; a major one and a minor one. In several of the crystalline preparations,

minor components were not detected. A proper linear response was obtained, so as to make quantitative work possible. This is shown in Plate 3(*a*) for a preparation without detectable minor component and in Plate 3(*b*) for one with an antigenically different minor component.

{In Plate 3(*a*) the running buffer was veronal. Lighter zones inside the precipitate cones suggest the presence of an additional antigen. However, veronal buffers were found regularly to lead to double-contoured precipitates with slowly moving antigen-antibody complexes. Such complexes are formed, for example, by α_2 -macroglobulin [such as is depicted in Fig. 4(*a*) of a paper by Clarke & Freeman (1968)] and by immunoglobulin G [such as is shown in a paper by Becker (1968) and discussed by Grubb & Skoog (1970)]. These double contours (sometimes referred to as 'tunnel effects') are not necessarily due to the presence of different antigenic species. They may be formed in certain conditions of voltage, temperature, buffer and antibody-antigen ratio or concentration. Additional work in altered conditions may be required to prove antigenic heterogeneity. Some features may suggest the presence of more than one antigen when inner cones show up. One of them is a clear and dark inner cone delineation (e.g. Plate 3*c*), or when the area inside the inner cone, or immediately inside its contour, is darker than outside it (e.g. Plate 3*b*). If an inner cone is due to a separate antigen, it should also yield a linear calibration curve (e.g. Plate 3*c*). Data presented in a paper by Laurell (1972) suggest that extreme caution should be taken in the qualitative interpretation of electroimmunoassay patterns. For Plate 3(*a*) (batch no. 90708) electroimmunoassay in 0.02M-sodium phosphate buffer, pH 6.0, proved that there is only one antigen present. For Plate 3(*b*) (batch no. 80115) two antigens were demonstrated by crossed immunoelectrophoresis (see, e.g., Plate 5*a*, after inhibition).}

Even when still working at pH 7.1, a perfect linearity for the minor component was obtained, although a certain amount of the antigen moves to the anode at that pH (Plate 3*c*). These facts indicate that, in our conditions, autodigestion can be disregarded.

Since 400 ng of antigen still gave a distinctly delineated cone of more than 2 cm length, the sensitivity can be estimated at less than 10 ng, without enhancement techniques. This brings the sensitivity of electroimmunoassay for subtilisin in the same range as for most other antigens. Further quantitative evaluation was not undertaken.

For electroimmunoassay, in contrast with crossed immunoelectrophoresis, no problem of resolution and band spacing exists. Further, at the base of cones formed in veronal buffer, pH 6.5, some reverse tailing is still visible (Plate 3*a*). Therefore 0.02M-sodium phosphate buffer, pH 6.0, was tried as an alternative for electroimmunoassay. The acceptability of this is

illustrated in Plate 3(b). In this system peak growth goes to completion at a higher rate.

Crossed immunoelectrophoresis

The combined application of the electroimmunoassay methodology and the improved electrophoresis system resulted in successful crossed immunoelectrophoresis. The resolution obtained can be judged in Plate 4. Inter-batch differences corresponding to the agarose-gel electrophoresis patterns are visible. Enzyme from batch no. 90919 (Plate 4, gel C and gel B position 2) contains a small amount of the minor antigen, which is not revealed in enzyme from batch no. 70-3 (Plate 4, gel A and gel B, position 1). These findings also reconfirm our earlier results, indicating that we are dealing with real molecular heterogeneity in the native enzyme (Verbruggen *et al.*, 1974; Verbruggen & Baeck, 1975).

The di-isopropyl phosphorofluoridate-inhibited enzyme shows, in the two-dimensional pattern, a change similar to that observed in regular electrophoresis: a bimodal peak replaces the single main component (Plate 5a). No explanation for this pattern change can be given yet. The introduction of the di-isopropyl group into the enzyme did not lead to losses of antigenic determinants, as detectable with our antibody preparation. This can be seen in Plate 5(b). Although the second-dimension run was not extended entirely to completion in this plate, the two preparations show merging precipitates.

Enzyme nomenclature

On the basis of these observations, and on those from Plates 4, 6(a), 6(b) and 6(c), it seems worth while to adapt the nomenclature that we introduced earlier (Verbruggen *et al.*, 1974). It cannot be excluded that antigenic differences would show up between bands termed ψ , χ and ϕ in earlier work, by using different types of antisera. The present work suggests, however, strong similarities between those main bands. Plate 6(c) represents a run at a high concentration of antigen (industrial material) and antibody, though at a less appropriate pH. It shows that the minor antigen can be analysed in a family of antigenically identical components. Three to four members of this family can be recognized. The slower-moving components were not revealed on agarose-gel electrophoresis, but show up on crossed immunoelectrophoresis. Thus we propose to speak of the ψ population for the main antigen, and of the ω population for the minor antigen population.

For both populations the members were numbered from cathode to anode. This way a new nomenclature is obtained, as shown in Plate 6(d), a slight idealization of Plate 6(c). In this drawing the former names have been placed in parentheses. It is probable that the trace component observed here as an addi-

tional third antigen is identical with the component termed ω_1 in our earlier work. The difference in the buffer system could explain the shift in relative mobility. If this is true, then this component too is a proteinase, as are the ψ and ω components. The heel protruding from ψ_1 towards the cathode can be termed ψ_0 , if it is later confirmed to be an additional isoenzyme.

Antigenic structure and isoenzyme integrity

The introduction of the di-isopropyl phosphorofluoridate group modifies the enzyme without affecting its antigenic integrity. The inhibited enzyme gives a reaction of complete identity with the non-inhibited one. Thus it is obvious that antigenic determinants in the immediate vicinity of the active site are not affected by modification with such a bulky group. Therefore one may deduce that the environment of the active site is devoid of determinants antigenic to rabbits. This is in line with results on phenylmethanesulphonyl fluoride-inhibited enzyme (Wicher & Dolovich, 1973).

A strong similarity between active-site areas of subtilisin and chymotrypsin was revealed by X-ray structure analysis (Kraut, 1971). The fact that both enzymes interact with the same serum inhibitors is in line with this. Therefore it seems logical that the active site of subtilisin is not antigenic, because the active site of chymotrypsin is not recognized as an antigen by rabbits producing chymotrypsin in their own bodies.

Stauffer (1973), working on the subtilisin Carlsberg molecule, has shown the existence of eight regions with antigenic activity towards the rabbit, which is a relatively high number for such a small molecule. Assuming that these sites are regularly dispersed over the surface of the molecule, with exclusion of its active-site area (as explained above), it must be concluded that the absence of partial identity reactions between the different enzyme bands is strong evidence in favour of the hypothesis that all the bands consist of intact species.

{The roughly spherical molecule has a diameter of 4.2 nm (42 Å) (Kraut, 1971). A sphere with a 4.2 nm (42 Å) diameter has a surface area of 55.42 nm² (5542 Å²). Assuming that each of the antigenic groups and the enzymic site occupy equal parts of the spherical surface, then 6.16 nm² (616 Å²) can be assigned to each group or site. Estimates for the surface area of an antigenic group range from 1 to 7 nm² (100 to 700 Å²) [Day (1972), p. 147]. We can also reason in an oversimplified way as follows. If the maximum contact surface area of 6.16 nm² (616 Å²) per site is considered circular, it has a diameter of 2.6 nm (26 Å). However, the cross-section diameter of a Fab fragment of immunoglobulin G is 3.5 nm (35 Å) [Day (1972), p. 108]. Even when not considering the enzymic site, the area occupied by eight Fab fragments pointing

to the subtilisin surface is $8 \times \pi \times 1.75^2 \text{ nm}^2$ ($8 \times \pi \times 17.5^2 \text{ Å}^2$) = 77.00 nm^2 (7700 Å^2) if no folding occurs. This is more than the available surface area. Thus we must conclude that the eight antigenic groups and the enzymic site together cover most of the molecule's surface area.}

Further, these findings suggest that the groups in the molecule whose variations cause the multiband pattern, have to be small in size so as not to influence antigenicity. The acid amide variations suggested by Verbruggen *et al.* (1974) seem to fulfil this requirement.

Drastic digestion of chain segments is in conflict with the findings reported here on the antigenic integrity of the area outside the active site. Verbruggen *et al.* (1974) showed that the multiband pattern can hardly be due to limited digestion. Enzymic activity with respect to gelatin and small esters was found in all bands (Verbruggen & Baeck, 1975). This and the fact that all bands bind the serum inhibitors suggests an unaltered and native topography around the active site as well. Both the active-site area and the antigenic part of the molecule outside the active-site area seem to be intact in all bands. Thus we seem to be dealing with a true isoenzyme system in the case of subtilopeptidase A.

Conclusions

A method for quantitative immunoelectrophoresis of subtilopeptidase A was developed. The enzyme, considered to be a pure and single protein until recently, was found to be a quite complex system of isoenzymes. All the findings reported are in line with the concept that each isoenzyme is an intact native species. The main species detected can be grouped in two populations of different antigenicity: ψ (major) and ω (minor). During the development work, several interactions of the enzyme with non-immunoglobulin components from serum were observed. A particular splitting of albumin was detected. These findings necessitated the use of purified immunoglobulins for good immunoelectrophoresis work.

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