Immunological Relationships of Bacterial L-Asparaginases

By SHOSHANA BASCOMB*

*Department of Biochemistry, Imperial College of Science and Technology,
London SW7 2AZ*

AND K. A. BETTELHEIM

*Department of Medical Microbiology, St Bartholomew's Hospital,
London EC1A 7BE*

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SUMMARY

Rabbit antisera against L-asparaginase preparations from *Escherichia coli*, *Erwinia carotovora*, *Citrobacter* sp. and *Chromobacterium violaceum* showed on immunoelectrophoresis that only the enzymes from *E. coli* and *Citrobacter* are immunologically related. Purified preparations had to be used to determine the immunological cross-reactions. Immunoelectrophoresis at different pH values yielded the zero mobility points of the enzymes. The activity of the *Er. carotovora* preparation was enhanced up to fourfold by homologous antiserum but not by normal sera. Heterologous antisera also enhanced, but only at a higher concentration. Less enhancement was observed for the other enzymes with antisera as well as with bovine serum albumin. Inhibition was not observed.

INTRODUCTION

L-Asparaginases have been used extensively for the treatment of experimental and human tumours (Capizzi, Bertino & Handschumacher, 1970). For most of these studies enzymes isolated from bacteria have been used, as L-asparaginases have been found in many species (Imada *et al.* 1973) and methods for large-scale purification developed (Wriston & Yellin, 1973; Bascomb *et al.* 1975). Repeated injection of L-asparaginase caused hypersensitivity in 5 to 20 % of patients (Cooney & Handschumacher, 1970). To avoid this allergic reaction and other long-term immunological effects, L-asparaginases with similar anti-tumour activities but of different antigenic specificities are needed to allow continued treatment of a responding but allergic patient. Therefore, when seeking further sources of L-asparaginase it is of paramount importance to establish, as early as possible, the immunological cross-reactions of the new enzyme with L-asparaginases in current use. The feasibility of determining the immunological cross-reactions of crude enzyme preparations is of particular interest.

Enzyme activity may be influenced by other proteins, particularly specific antibody. With most enzymes, interaction with specific antibody inhibits enzyme activity, but enhancement has also been described (Cinader, 1967; Pollock, Fleming & Petrie, 1967; Dyke, 1967). Lee & Bridges (1968) reported that various non-immune animal and human sera enhanced the L-asparaginase activity of *E. coli* extracts, but L-asparaginase of guinea-pig serum was enhanced only by human and sheep sera. Ho & Jones (1969) showed that the activity of a purified *E. coli* enzyme was increased by normal sera or by albumin. They concluded that

* Present address: Department of Bacteriology, St Mary's Hospital Medical School, London W2 1PG.

the effect was non-specific and that bovine serum albumin enhanced the L-asparaginase activity by coating the surface of the reacting vessel, thus preventing denaturation of the enzyme by adsorption. However, MacLennan, Wade & Hawkins (1971) showed that homologous antisera inhibited the *E. coli* and *Erwinia carotovora* enzymes specifically.

This paper describes the immunological relationship between purified L-asparaginases from five different bacterial species and the effects of antibody on the *in vitro* activity of these enzymes.

METHODS

Organisms. Bacterial strains from the culture collection of the Biochemical Department of Imperial College were used, and their identity is given in the text.

Enzyme preparations. Commercial preparations of L-asparaginase from *E. coli* were obtained from Bayer-Leverkuse (Farbenfachen Bayer AG, Germany) and those from *Er. carotovora* from the Microbiological Research Establishment, Porton.

Purified L-asparaginase from *Citrobacter* c6 was obtained by the method described by Bascomb *et al.* (1975). A similar method was used for the preparation of enzymes from *Chromobacterium violaceum* A153 and *Proteus vulgaris* A108.

Crude extracts of *Citrobacter* c6 were prepared as follows: The organisms were grown in a 60 l fermenter (Bascomb *et al.* 1975), harvested by centrifuging (Sharples No. 6 tubular bowl centrifuge at a rate of 100 l/h at 4 to 5 °C) and resuspending the pellet in potassium borate buffer (50 mM, pH 8.0). The chilled (4 to 5 °C) resuspended bacteria were ruptured by passing through an APV Manton-Gaulin sub-micron disperser model K3 operated at a gauge pressure of 5×10^7 Pa bar and at a rate of 250 l/h. The ruptured bacteria were centrifuged for 15 min at 4 °C at 38000 g in an MSE high speed centrifuge, and the supernatant, which contained the enzyme activity, stored at -20 °C.

Assay of L-asparaginase activity and protein. Enzyme activity was measured by three different methods. Method 1: an automated procedure, based on estimating the ammonia released from the substrate after 15 min incubation with the enzyme preparation (Bascomb *et al.* 1975). This was used for the quantitative determination of enzyme activity. A control channel measuring ammonia content of the enzyme preparation and a channel measuring its protein content were included in the system. Method 2: an automated procedure with reaction time of 1 min (Bascomb *et al.* 1975). This was used for the *in vitro* assay of enzyme-antibody mixtures. Method 3: qualitative estimation of enzyme activity on immuno-electrophoresis slides. After electrophoretic separation, a strip of Whatman No. 1 filter paper soaked in 33 mM L-asparagine in physiological saline was laid on the gel, and incubated in a moist chamber in a 37 °C incubator for 10 min. The filter paper strip was then dried at 50 °C, dipped in a 2% (w/v) ethanolic solution of ninhydrin and dried at 50 °C for 10 to 15 min. Enzyme activity was indicated by a blue coloration (aspartic acid), while the unchanged asparagine was brown. The size of the blue area was used for a rough estimation of enzyme activity.

Antibody production. New Zealand white rabbits (2 to 3 kg) were used. Blood was obtained before injection and the serum found free of antibodies to any of the asparaginases tested. These 'pre-inoculation' sera were used as controls in some of the experiments. One week after bleeding, 0.5 ml of enzyme preparations (10 mg protein/ml sterile saline) plus an equal volume of Freund's complete adjuvant were inoculated intramuscularly into one hind leg. Five weeks later 0.25 ml of enzyme preparation (1 mg protein/ml) was inoculated intramuscularly into each hind leg and 0.1 ml of the same preparation intravenously. The rabbits were bled 1 week afterwards. The blood was allowed to clot at room temperature, and the

Fig. 1. Cross-reactivity of bacterial L-asparaginases with antisera. Electrophoresis was in 20 mm-potassium phosphate buffer pH 8.0. (a) Immunoelectrophoresis of *Er. carotovora* (Er), *Citrobacter* sp. (Ci) and *E. coli* (Es) enzymes with rabbit antisera against *Er. carotovora* (er) and *E. coli* (es) enzymes. (b) L-Asparaginase activity of enzymes on slide (a) as detected by method 3. (c) Immunoelectrophoresis of *Citrobacter* sp. and *E. coli* enzymes with rabbit antisera (ci) against *Citrobacter* sp. enzyme preparations from two batches. (d) L-Asparaginase activity of enzymes on slide (c) as detected by method 3.

serum was removed by pipette and centrifuged at 300 g for 30 min to remove the remaining red cells. All sera were stored at -20°C .

Electrophoresis. Potassium phosphate buffers (20 mM) of different pH values, as described by Bettelheim & Taylor (1970), were used in all studies. Standard Shandon equipment was employed both for cutting antigen wells and antiserum troughs in the gels, which contained 1.2 % (w/v) Ion-Agar No. 2 (Oxoid), and for electrophoresis. A constant voltage of 200 V was maintained for 1 h, during which time the current increased from 30 to 60 mA. After electrophoresis the position of the enzymes on the slide was located by method 3. Antisera were then placed in the troughs, and the slides incubated in a humid chamber at room temperature for 24 h before washing and staining (Bettelheim & Taylor, 1970).

RESULTS

Immunoelectrophoresis with homologous and heterologous sera

Antisera prepared against enzyme preparations from *Citrobacter* c6, *Chr. violaceum* A153, *Er. carotovora* and *E. coli* were each tested against all four enzymes by immunoelectrophoresis at different pH values. A typical slide is shown in Fig. 1(a). *Erwinia carotovora* and *E. coli* enzymes gave only one precipitin line with their homologous antisera. The position of this line always coincided with the position of asparaginase activity on the filter paper strip (Fig. 1b). When *Citrobacter* and *Chr. violaceum* enzymes were tested against their homologous sera, a number of precipitin lines were observed (Fig. 1c). Only one of these lines coincided with the position of enzyme activity on the filter paper. For immunological comparisons the position of the enzyme was determined as that precipitin line which was closest to enzyme activity. When the *Citrobacter* preparation was tested against *E. coli* antiserum, only one line was observed. Its position coincided at all pH values with the enzyme line detected with the *Citrobacter* antiserum. Conversely, when the *E. coli* enzyme was tested against the *Citrobacter* serum, only one line was observed in the same position as detected with the *E. coli* antiserum. An immunological relationship was detected only between the *E. coli* and *Citrobacter* enzymes.

Fig. 2. Electrophoretic mobility of bacterial L-asparaginases. Electrophoresis was performed with 1.2 % (w/v) ion agar (Oxoid No. 2) in 20 mM potassium phosphate buffers. Source of enzymes: ■, *Citrobacter* sp.; ▲, *Chr. violaceum*; ●, *E. coli*; ○, *Er. carotovora*.

The possibility of detecting cross-reactivity in crude preparations from *Citrobacter* was investigated with antisera against the *E. coli* and *Citrobacter* enzymes. The ruptured bacteria and supernatant of *Citrobacter* were subjected to electrophoresis at pH 6.0 and enzyme activity could be detected in both by method 3. Although clear precipitin lines were observed when each antiserum reacted with its homologous antigen, the two crude *Citrobacter* preparations reacted only faintly with *Citrobacter* antiserum and barely visibly with the *E. coli* antiserum. Therefore, it would appear that crude extracts do not permit the detection of immunological relationships of L-asparaginases and purer enzyme preparations are necessary.

A partially purified enzyme preparation from *P. vulgaris* A108 was tested against all four antisera at pH 8.0. It formed a precipitin line only with the *E. coli* antiserum at a position which coincided with that of the enzyme activity.

Effect of pH on electrophoretic mobility of enzymes

The electrophoretic mobilities of the four purified L-asparaginases were studied at pH values ranging from 5.0 to 9.0 at 0.5 pH unit intervals (Fig. 2). Enzyme mobility was estimated by measuring the distance between the antigen well and the point on the precipitin line nearest to the antiserum trough (Fig. 1a). Each enzyme was tested against its homologous antiserum at every pH on at least three separate slides on two separate occasions.

Table 1. *Effect of added protein on activity of L-asparaginase from different bacteria*

Enzymes were mixed with protein in 50 mM-potassium borate buffer pH 8.0 (1:9, v/v), incubated for 30 min at 30 °C and assayed by method 1. Enzyme activity of control samples for *Citrobacter*, *Er. carotovora* and *E. coli* enzymes was 4, 1.5 and 14 i.u./ml, respectively.

Protein added		L-Asparaginase activity (% of control)		
Type	Amount (mg/ml)	Source of enzyme:		
		<i>Citrobacter</i>	<i>Er. carotovora</i>	<i>E. coli</i>
None (control)		100	100	100
Rabbit pre-inoculation serum	5.3	139	NT	NT
<i>Citrobacter</i> antiserum	5.6	126	152	135
<i>Er. carotovora</i> antiserum	5.8	143	420	130
<i>E. coli</i> antiserum	6.9	93	173	130
Bovine albumin fraction V	1.0	149	146	136

NT, not tested.

Table 2. *Effect of concentration of antiserum and incubation period on activity of Er. carotovora L-asparaginase*

Enzyme (1.2 i.u./ml) was mixed in 50 mM-potassium borate buffer pH 8.0, incubated with slight agitation at 37 °C for the stated period and assayed at 1/250 dilution by method 2.

Concentration of antiserum in incubated mixture (mg protein/ml)	L-asparaginase activity (% of control)		
	Incubation time (h)		
	0	1	2.5
0	100	35	36
<i>Er. carotovora</i> antiserum			
0.29	147	147	133
0.58	266	269	260
2.90	ASR	ASR	ASR
5.80	ASR	ASR	ASR
<i>Chr. violaceum</i> antiserum			
0.0	44	40	44
0.3	44	66	48
0.6	56	57	53
3.0	188	236	214
6.0	ASR	ASR	ASR

ASR, Above sensitivity range of instrument.

Effects of antibody on enzyme activity

Enzymes of *Citrobacter*, *E. coli* and *Er. carotovora* were diluted in 0.9 ml potassium borate buffer (50 mM, pH 8.0) with 0.1 ml of the various sera or albumin, incubated with gentle agitation for 30 min at 30 °C, and assayed for enzyme activity by method 1. Enzyme activity was detected in all enzyme preparations but not in any of the added proteins. Free ammonia was not detected in any of the sera or the albumin. All three enzymes (Table 1) showed some increase in activity with added protein; activity of the *Er. carotovora* enzyme in homologous antiserum increased more than fourfold.

A more detailed study of the effect on activity of different concentrations of enzyme and antibody, and of the duration of incubation with antiserum, was investigated with the

Er. carotovora enzyme by the more sensitive method 2. The enzyme was diluted as before with 0.005, 0.01, 0.05 and 0.1 ml of its homologous serum (equivalent to 0.3, 0.6, 2.9 and 5.8 mg protein/ml, respectively), and the mixture assayed immediately and after 1 and 2.5 h gentle agitation at 37 °C. The activity of enzyme agitated without serum was reduced within 1 h of incubation (Table 2). Addition of homologous antiserum increased enzyme activity at zero time even at the lowest dilution used (0.3 mg protein/ml agitated mixture, or a final dilution in the assay mixture of 0.001 mg protein/ml) and protected the enzyme from deterioration during incubation at 37 °C. The deterioration in activity of enzyme dilutions without added protein was detected in further experiments even at room temperature within 10 min of the dilution of the enzyme. Addition of heterologous antisera (Table 2) increased enzyme activity, but this increase was smaller and apparent only when 0.05 ml of serum (3.0 mg protein/ml) was added. At such concentration of antiserum, enzyme activity did not deteriorate during 2.5 h incubation at 37 °C. Effects of antisera on activity of *Citrobacter* and *E. coli* enzymes were generally much less and rather variable.

DISCUSSION

The immunoelectrophoresis studies confirmed that the *Er. carotovora* and *E. coli* enzymes are serologically unrelated (MacLennan *et al.* 1971), and showed that the L-asparaginase from *Chr. violaceum* is again different. The *Citrobacter* enzyme is related to that from *E. coli*, as shown by the precipitin lines developed by each enzyme against the other antiserum. Nevertheless, the mobilities and zero mobility points (see below) of these two enzymes are slightly different, suggesting some structural differences. This is also shown by the molecular weights of these two enzymes, 120 000 to 127 000 daltons for the *E. coli* enzyme (as quoted by Wriston & Yellin, 1973) and 166 000 daltons for that from *Citrobacter* (Bascomb *et al.* 1975). *Escherichia* and *Citrobacter* are closely related genera (Edwards & Ewing, 1972) within the Enterobacteriaceae, while *Erwinia*, although in the same family, is less closely related to these two. *Chromobacterium* is not in the Enterobacteriaceae. A precipitin line was also observed with the *E. coli* serum and the *P. vulgaris* preparation. This serological relatedness was less expected, as *Proteus* and *Escherichia* belong to two different tribes of Enterobacteriaceae. These results are similar to those of Cocks & Wilson (1972), who studied serological relationships between alkaline phosphatases from different bacterial taxa and concluded that the immunological relationships they observed corresponded with the accepted taxonomy of the groups.

By comparing the mobilities of the enzymes on the slides after electrophoresis at different pH values, a zero mobility point can be established for each enzyme at any set of specified conditions for electrophoresis. Theoretically, this point is related to the isoelectric point of the enzyme but is affected by the electroendosmosis specific to the electrophoretic conditions used. In comparison with the established methods of isoelectric focusing this method is much cheaper to perform and can be combined with serological and enzyme activity studies. It is, therefore, relevant to compare the isoelectric points found by isoelectric focusing (Robinson, 1972) with the zero mobility points found by our method. The *Er. carotovora* values of isoelectric focusing varied from 7.7 to 8.35 and those for the *E. coli* (Bayer) preparation varied from 4.95 to 5.5. The points of zero mobility were 8.2 and 6.2 respectively (Fig. 2). The agreement in *Erwinia* is encouraging, while the discrepancy in the *E. coli* enzyme may be due to batch differences or the nature of the buffer used, or to electroendosmosis effects, as our method employed the rather highly charged agar for the gel matrix.

The non-specific enhancement of activity of the *E. coli* and *Citrobacter* enzymes confirms

the results of Ho & Jones (1969). MacLennan *et al.* (1971) showed that concentrations of homologous antiserum as low as 1/25000 partially inhibited the *E. coli* and *Er. carotovora* enzymes and that increasing the concentration of antiserum to 1/8 increased the inhibition. However, our results do not confirm this finding. With the *E. coli* enzyme (Table 1), homologous and heterologous sera had similar effects of increasing the enzyme activity by 30 to 36 %. With the *Er. carotovora* enzyme, homologous antiserum at concentrations ranging from 1/200 to 1/10 caused an increase in enzyme activity (up to fourfold at 1/10). Neither albumin at 0.1 mg/ml provided such a stimulation, nor did the non-immune or heterologous sera, although the latter gave some enhancement of activity at higher concentrations.

The enhancement of L-asparaginase activity by specific antibody which we found *in vitro*, may also occur *in vivo*. In such situations the determination of the clearance rate of the enzyme from the bloodstream as currently performed on serum might give misleadingly low results. If specific antibodies cause the attachment of enzyme to lymphocytes or other cells of the blood, most of the enzyme may not only remain undetected in the precipitated clot but also be enhanced.

The differences between our findings and those of MacLennan *et al.* (1971) may be explained on the basis of variation in the type of antibody response induced in individual rabbits. Cinader (1967) reported that five out of six rabbits produced inhibitory antibodies to ribonuclease, while the sixth consistently produced an antibody which increased the activity of the enzyme. Such variation in type of antibody response may contribute to the variability of patient response to L-asparaginase treatment.

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