The Sequential Limited Degradation of Bovine Myelin Basic Protein by Bovine Brain Cathepsin D*  

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Bovine myelin basic protein (BP) microheterogeneous components were isolated and exposed to homogeneous bovine brain cathepsin D purified by affinity chromatography on immobilized pepstatin. The extent of the reaction was followed by polyacrylamide gel electrophoresis at pH 8.8, and the reaction products were separated by column chromatography on carboxymethyl-cellulose and Sephadex following which the BP peptides were characterized by amino acid analysis and partial sequence analysis. Components one and three were degraded with the initial site of cleavage at the Phe-Phe bond at residues 42 and 43 to generate peptides 1-42 and 43-169. With more prolonged exposure to enzyme, peptide 1-42 was degraded to form peptide 1-36 and peptide 43-169 was degraded to form peptides 43-88, 89-169, and 92-169 as well as smaller amounts of peptides 43-89 and 43-91. Pepstatin inhibited the initial cleavage of BP by cathepsin D. Microheterogeneous components two, four, and five showed similar patterns of fragmentation to yield bands with the migration of peptides 1-36, 43-88, and 89-169 or 92-169. Peptides 43-169, 89-169, and 92-169 had a decreasing cathodal migration progressing from component one to five. These findings demonstrate the sequential but limited cleavage of BP by brain cathepsin D. The effects of the enzyme on the microheterogeneous components of the molecule in forming fragments of different charge characteristics suggest that the processes regulating microheterogeneity may influence the outcome of degradation of BP by brain cathepsin D and possibly other proteinases.

Myelin encephalitogenic or basic protein, which accounts for approximately 30% of myelin proteins in the central nervous system (CNS) (1), has a monomeric molecular weight of 18,500 and consists of 169 amino acid residues (2, 3). The total amino acid sequence has been determined for human (4) and bovine (2, 3) BP1 and the small BP of the rat (5), and the partial sequence is known for several other species (6). The amino acid distribution gives to the molecule a number of hydrophobic and hydrophilic domains (7). Chemical information available on BP has revealed several molecular modifications which may be important in the biosynthesis and metabolism of BP. These include microheterogeneity, best demonstrated at alkaline pH (8, 9), which appears to be due to a combination of processes resulting in partial phosphorylation at threonine 97 and serine 164 and partial deamidation of glutamino acids at positions 102 and 146 (10).

Although the extensive studies of BP have been a result of its ability to induce experimental allergic encephalomyelitis (EAE) (11), BP has other well recognized immunological properties of stimulating cellular immunity (12) and antibody formation (13) which may occur independently of the appearance of EAE. In addition to the triggering of immunological events, BP or its fragments may also affect certain biological phenomena such as neuroelectric activity (14) and fibroblast proliferation (15). A number of molecular regions of BP have been demonstrated to contain the major encephalitogenic site, depending on the species involved (16), and the antigenic regions for cellular and humoral immunity are spread throughout the molecule (17, 18). The areas of the molecule responsible for the biological, nonimmunological effects are less well established. With these possible molecular influences, it becomes important to determine what BP fragments may occur in vivo.

Brain contains an acid proteinase (19) which has the features of cathepsin D (EC 3.4.4.23) (20). Changes of brain cathepsin D activity during CNS development (21) and its increased level in inflammatory demyelination of the CNS such as in brains of animals with EAE (22) and tissue lesions of persons with multiple sclerosis (23) suggest that this enzyme is involved in the normal metabolism and pathological degradation of CNS proteins. BP is known to be very sensitive to degradation by this enzyme (3, 24-26); however, information on the sites of enzymatic cleavage is not in agreement. The present study was undertaken to define further the digestion of BP by brain cathepsin D, particularly how it might relate to the appearance of fragments of BP in cerebrospinal fluid during active demyelination in persons with multiple sclerosis (27). The results demonstrate a limited but stepwise cleavage of BP.

**EXPERIMENTAL PROCEDURES**

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† Medical Investigator of the Veterans Administration.

‡ The abbreviations used are: BP, myelin basic or encephalitogenic protein; CNS, central nervous system; EAE, experimental allergic encephalomyelitis.

1 Numbering of residues in bovine BP is based on the reported sequence of 170 amino acids (2) with the corrected deletion of the serine residue at position two (3).

2 Materials—Carboxymethyl-cellulose (CM52) was obtained from Whatman, Sephadex from Pharmacia, Bio-Gel P-2 from Bio-Rad, molecular weight markers (ovalbumin, chymotrypsinogen A, and cytochrome c) from Schwarz/Mann, and pepstatin A from Peptide Institute, Inc. (Osaka, Japan). Bovine brain was obtained from a local slaughterhouse.

Purification of BP—BP was isolated from a pH 3 extract of...
delipidated bovine brain using ion exchange chromatography on CM-
cellulose at pH 10.5 (18, 28). The multiple peaks of BP eluting with
the NaCl gradient were designated as microheterogeneous compo-
nents one to five with component one being the last to elute (9). The
individual BP components were desalted on Sephadex G-25 in 0.5%
acetic acid and lyophilized. Individual components were rechroma-
tographed separately over CM-cellulose and desalted under identical
conditions and, when necessary as indicated by disc gel electropho-
resis at pH 2.5 (28), further purified by filtration through a column
(2.6 x 95 cm) of Sephadex G-150 equilibrated with 0.2 M ammonium
bicarbonate.

Purification of Brain Cathepsin D—Cathepsin D was purified to
appear homogeneity from an extract of bovine brain by means of
affinity chromatography on immobilized pepstatin. The details of the
preparation and characterization of the enzyme have been described
(29). The amount of enzyme to be used was based on protein concen-
tration as determined by the Lowry procedure (30). Several different
preparations of brain cathepsin D were used throughout the course
of these investigations so that there was some small variation in
specific activity from the previously described specific activity of
approximately 5500 units/mg of protein (29).

Digestion of BP with Brain Cathepsin D—For analytical poly-
acrylamide disc gel electrophoretic studies, 300 to 400 μg of BP or BP
peptide were dissolved in 1 ml of 0.05 M ammonium acetate, pH 3.5,
with or without varying amounts of enzyme and incubated at 37°C in
a shaking water bath. When incubation mixtures contained pepstatin,
this proteolytic inhibitor was mixed with 0.05 M ammonium acetate,
pH 3.5, and added in a volume of 50 μl. Incubation mixtures were
frozen, lyophilized, and dissolved in 100 μl of sample buffer for disc
gel studies.

For preparation of BP peptides to be isolated by chromatographic
procedures, BP was dissolved at a concentration of 5 mg/ml in 0.05
M ammonium acetate, pH 3.5, and incubated with enzyme at 37°C.
Following the incubation period, samples were frozen and lyophilized
prior to CM-cellulose chromatography.

Column Chromatography—BP peptides, prepared by treatment
with brain cathepsin D, were separated by ion exchange chromatog-
raphy on CM-cellulose equilibrated with 0.02 M ammonium bicarbon-
ate, pH 7.5 (18). The column effluent was monitored by absorption at
225 nm. After the small peak of unretarded material was eluted from
the column, a NaCl gradient of 0 to 0.3 M NaCl (total volume of 1
liter) in the same buffer was begun. Fractions from individual peaks
were pooled, lyophilized, and the product desalted on Bio-Gel P-2 in
0.5% acetic acid. Desalted samples were again lyophilized and then
subjected to gel filtration over a column (1.5 x 90 cm) of Sephadex
G-50 (superfine) equilibrated with 0.2 M ammonium bicarbonate and
monitored by absorption at 225 nm. The columns of Sephadex G-50
were calibrated with ovalbumin (M, = 45,000), chymotrypsinogen A
(M, = 25,000), and cytochrome c (M, = 13,000). As subsequently
determined by studies of amino acid composition, BP fragments
collected from the column at points usually indicative of a considerably
greater molecular weight. Similar behavior of BP and its peptides has
been reported by others (31), and is presumed to be due to the
nonglobular conformation and electrostatic charge of BP. Appropriate
fractions from the gel filtration were pooled, lyophilized twice, and
analyzed for amino acid composition and by polyacrylamide disc gel
electrophoresis at pH 8.8 (26).

Polyacrylamide Disc Gel Electrophoresis—Purity of BP and BP
microheterogeneous components was monitored by polyacrylamide
disc gel electrophoresis at pH 2.5 or pH 10.6 in the presence of urea
(28). BP, BP digests, BP peptides, and digests of BP peptides were
subjected to electrophoresis in 10% polyacrylamide disc gels (6 x 65
mm) (26). This system of gel electrophoresis was selected to follow
the extent of the reaction because it permits a clear separation of most
of the BP peptides formed. Twenty to forty micrograms of protein in
10 μl of sample buffer were applied per gel and migration
was toward the cathode. One hundred micrograms of cytochrome c
alone were applied to one gel to provide a visual marker of the
electrophoretic migration. The jacketed electrophoresis chamber was
cooled by circulating tap water. Electrophoresis was terminated when
the cytochrome c was within 1 cm of the cathodal end of the gel.

Characterization of BP Fragments—Amino acid analysis
was performed on samples hydrolyzed under an atmosphere of N2 in
constant boiling 6 N HCl at 108°C for 24 h. An automatic amino acid
analyzer (Beckman) was utilized with a single-column method previously
described (32). No correction factors were used for losses of
labile amino acids or for the incomplete release of valine.

Automatic Edman degradations were performed with a Beckman
Sequencer, model 890C following the principles of Edman and Begg
(33). The Slow Peptide-Dimethylallylamine (071472) Program of
Beckman Instruments was employed. The phenylthiohydantoins
study at pH 8.8 of the degradation of bovine BP microheterogeneous
components three (a) and one (b), by brain cathepsin D with increasing
lengths of incubation. BP was mixed with brain cathepsin D at a
ratio of 100:1 and incubated for 0 (1), 30 (2), 60 (3), 120 (4), 240 (5),
and 480 (6) min at 37°C. Letters to the right for b correspond to the
fractions designated in Figs. 4 and 5. Forty micrograms of protein per
gel. Cathode at bottom.
FIG. 4. CM-cellulose chromatography (2.6 × 18 cm) in 0.02 M NH₄HCO₃ of 97 mg of bovine BP microheterogeneous component one digested with brain cathepsin D at a protein to enzyme ratio of 180:1 at 37°C for 30 min. The NaCl gradient was begun at tube 25 and was from 0 to 0.3 M NaCl. Horizontal bars indicate the tube contents (8.3 ml/tube) pooled together.

FIG. 5. Polyacrylamide disc gel electrophoretic study at pH 8.8 of the BP peptides prepared by the degradation of bovine BP microheterogeneous component one by brain cathepsin D. Gels contain BP component one incubated alone (I) or with brain cathepsin D at a protein to enzyme ratio of 180:1 (2) at 37°C for 240 min and fragments B (peptide 43-88) (3), D (peptide 1-36) (4), E (peptide 1-42) (5), G (peptide 92-169) (6), and H (peptide 89-169) (7) obtained by CM-cellulose chromatography (see Fig. 4) followed by gel filtration on Sephadex G-50 (superfine). Forty micrograms of protein per gel. Cathode at bottom. Tables I and II contain amino acid analysis data on these peptides.

were identified by high pressure liquid chromatography (Waters Associates, Milford, MA) on two prepacked columns (0.4 × 30 cm) of μBONDPAK C₈ using acetonitrile/sodium acetate, pH 4.0, at a flow rate of 1.9 ml/min (34).

RESULTS

Purification of BP—Bovine BP eluted from CM-cellulose at pH 10.5 as five distinct peaks (Fig. 1) which were shown by polyacrylamide gel electrophoresis at pH 2.5 in the presence of urea to represent a single major band with a variable and slight admixture of the BP polymer band (35). Electrophoresis at pH 10.6 in the presence of urea demonstrated the microheterogeneity of BP and that BP microheterogeneous components one and three, which were to be used for the preparation of BP peptides, did not contain other BP components.

Degradation of BP—Following extensive treatment (protein-enzyme ratio of 100:1 with incubation at 37°C for 8 h) with brain cathepsin D, the five microheterogeneous compo-

ments yielded two major and one minor peptide bands in common (Fig. 2). The faint anodal band designated A was usually less apparent in the digest of component one than in that from components two to five. Consistent and more marked differences were noted in the cathodally migrating material which showed a decrease in electrophoretic mobility declining from components one to five. These differences were clearly illustrated in components one and three; so these BP

FIG. 6. CM-cellulose chromatography (2.6 × 18 cm) in 0.02 M NH₄HCO₃ of 75 mg of bovine BP microheterogeneous component one digested with brain cathepsin D at a protein to enzyme ratio of 1200:1 at 37°C for 60 min. Adherent peptides were eluted with 0 to 0.3 M NaCl gradient in 0.02 M NH₄HCO₃. Horizontal bars indicate the tube contents (9 ml/tube) pooled together.

FIG. 7. Polyacrylamide disc gel electrophoretic study at pH 8.8 of the more extensive digestion by brain cathepsin D of BP peptide 1-42 derived from microheterogeneous component one. Gels contain BP component one (I), BP component one incubated with brain cathepsin D at a protein to enzyme ratio of 200:1 for 120 min (2), peptide 1-42 (Peak E in Fig. 4) (3), and peptide 1-42 incubated with brain cathepsin D at a protein to enzyme ratio of 200:1 for 30 (4) and 120 min (5). Twenty micrograms of protein per gel on Gels 2 to 5. Cathode at bottom.
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The chromatographic profile changed with more prominent C and E peaks and with the change in the protein peak eluting in the region of G and H (Fig. 6). This corresponded to the pattern on gel electrophoresis after less extensive enzyme treatment of BP (Fig. 3B, Gel 4). In order to investigate the relationship

species were studied further in a timed incubation with brain cathepsin D (Fig. 3). Both components showed similar temporal changes, but the banding pattern was more clearly resolved in the digestion of component one. Within 30 min, BP had been cleaved into two major bands having a more rapid cathodal migration than intact BP. After longer periods of incubation, these bands, particularly the one nearer intact BP, became less dense or disappeared. Simultaneously, other bands appeared leading to the banding pattern as previously noted in Fig. 2. If the time of incubation was held constant and the relative amount of brain cathepsin D varied over a 10-fold range, a similar appearance and transition of banding patterns on gel electrophoresis indicated that brain cathepsin D produced a limited, but sequential, degradation of BP initially involving larger BP peptides which could be further digested with more prolonged treatment with the enzyme. Studies were then undertaken to characterize the peptides formed.

Preparation and Characterization of BP Peptides—Larger amounts of BP were digested with brain cathepsin D, and the peptides formed were isolated on CM-cellulose at pH 7.5 (18). With a low protein to enzyme ratio, five major peptides were identified and separated (Fig. 4). They had electrophoretic migrations (Fig. 5) identical with bands B, D, E, G, and H shown in Fig. 3B. As indicated by the chromatographic patterns, the digestion of BP by enzyme in larger volumes appears to have been more extensive than in the small reaction mixture used to prepare material for gel electrophoretic studies. The reasons for these differences are unclear at this time. By lowering the relative amount of enzyme present, the chromatograms were studied further in a timed incubation with brain cathepsin D (Fig. 3). Both components showed similar temporal changes, but the banding pattern was more clearly resolved in the digestion of component one. Within 30 min, BP had been cleaved into two major bands having a more rapid cathodal migration than intact BP. After longer periods of incubation, these bands, particularly the one nearer intact BP, became less dense or disappeared. Simultaneously, other bands appeared leading to the banding pattern as previously noted in Fig. 2. If the time of incubation was held constant and the relative amount of brain cathepsin D varied over a 10-fold range, a similar appearance and transition of banding patterns on gel electrophoresis indicated that brain cathepsin D produced a limited, but sequential, degradation of BP initially involving larger BP peptides which could be further digested with more prolonged treatment with the enzyme. Studies were then undertaken to characterize the peptides formed.

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Amino acid analysis of peptides of bovine BP component one

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Peptides</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histidine</td>
<td>1.9 (2)b 2.1 (2) 2.8 (3) 11.2 (11) 8.5 (8) 7.9 (7)</td>
</tr>
<tr>
<td>Arginine</td>
<td>3.0 (3) 3.1 (3) 5.3 (5) 6.9 (7) 1.9 (2) 2.3 (2)</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>5.3 (5) 5.6 (6) 3.7 (4) 11.1 (11) 7.5 (7) 6.8 (7)</td>
</tr>
<tr>
<td>Threonine</td>
<td>2.9 (3) 2.6 (3) 3.9 (4) 8.4 (8) 4.2 (4) 3.3 (3)</td>
</tr>
<tr>
<td>Serine</td>
<td>1.8 (2) 1.7 (2) 1.9 (2) 5.1 (5) 3.0 (3) 2.8 (3)</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>4.2 (4) 5.2 (5) 2.5 (3) 14.3 (13) 8.9 (10) 8.8 (10)</td>
</tr>
<tr>
<td>Proline</td>
<td>1.9 (2) 1.7 (2) 3.7 (4) 7.9 (8) 4.1 (4) 4.2 (4)</td>
</tr>
<tr>
<td>Glycine</td>
<td>1.9 (2) 1.7 (2) 3.6 (4) 10.4 (10) 5.9 (6) 5.4 (6)</td>
</tr>
<tr>
<td>Alanine</td>
<td>2.0 (2) 3.2 (3) 6.4 (7) 21.9 (22) 14.8 (15) 14.1 (15)</td>
</tr>
<tr>
<td>Valine</td>
<td>4.7 (5) 5.0 (5) 3.8 (4) 9.1 (9) 5.2 (6) 5.0 (5)</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.0 (0) 0.0 (0) 1.4 (2) 2.3 (3) 0.7 (1) 0.6 (1)</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>1.0 (1) 0.9 (1) 0.0 (0) 1.5 (2) 1.9 (2) 1.5 (2)</td>
</tr>
<tr>
<td>Leucine</td>
<td>3.4 (3) 4.3 (4) 1.0 (1) 6.0 (6) 5.5 (5) 5.2 (5)</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>1.0 (1) 0.9 (1) 1.0 (1) 3.2 (3) 2.2 (2) 2.1 (2)</td>
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<tr>
<td>Phenylalanine</td>
<td>1.0 (1) 1.8 (9) 2.0 (2) 6.1 (6) 4.0 (4) 3.0 (3)</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>ND* (1) ND (1) ND (1)</td>
</tr>
</tbody>
</table>

*Letters in the column headings have the same designation as that used for bands seen on gel electrophoresis and peaks present on column chromatography. Letters in parentheses following the letters indicate the appropriate BP peptide. Numbers in parentheses following the letters indicate the number of residues based on the known sequence of bovine BP (2, 3).

ND, not determined.

Table II

NH2-terminal sequence of fragments of BP component one

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Phe - Gly - Ser - Asp - Arg - Gly</td>
</tr>
<tr>
<td>B</td>
<td>Blocked NH2- terminal</td>
</tr>
<tr>
<td>D</td>
<td>Blocked NH2- terminal</td>
</tr>
<tr>
<td>E</td>
<td>Phe - Lys - Asn - Ile - Val - Thr</td>
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<tr>
<td>G</td>
<td>Ile - Val - Thr - Pro - Arg - Thr - Pro</td>
</tr>
<tr>
<td>H</td>
<td>Blocked NH2- terminal</td>
</tr>
<tr>
<td>GH</td>
<td>Phe - Gly - Ser - Asp - Arg - Gly - Ala - Pro</td>
</tr>
<tr>
<td>GH</td>
<td>Phe - Lys - Asn - Ile - Val - Thr - Pro</td>
</tr>
<tr>
<td>Ile</td>
<td>Val - Thr - Pro - Arg - Thr - Pro</td>
</tr>
</tbody>
</table>

*Letters have the same designation as that used for bands seen on gel electrophoresis and peaks present on column chromatography. Numbers indicate position in the bovine BP molecule (2, 3).

between the BP peptides generated at early and late phases of degradation, the BP peptides formed early were isolated and subjected to a second digestion with enzyme. On further incubation of peptide E with enzyme, peptide D was formed (Fig. 7) and treatment of peptide GH led to the formation of peptides A, B, and G or H (Fig. 8). Exhaustive treatment of peptides B and D with cathepsin D at a protein to enzyme ratio of 50:1 for 16 h at 37°C produced no further degradation of these peptides.

The peptides isolated by CM-cellulose chromatography were subjected to gel filtration over Sephadex G-50 (superfine) (Fig. 9) and the recovered fractions were characterized by amino acid composition (Table I) and partial sequence analysis (Table II). All peptides obtained from component one were so studied, except for Peaks A and C, for which insufficient material was available. In all preparations of B, a small amount of A could be detected (Fig. 5) even after gel filtration. With this chemical information, the peptides formed could be unequivocally identified. By these methods, Band B was identified as BP peptide 43–88, Band D as peptide 1–36, Band E as peptide 1–42, Band G as 92–169, and Peak H as peptide 89–169. The latter could be resolved on CM-cellulose chromatography (Fig. 4) but merged with Band G on gel electrophoresis at pH 8.8 (Figs. 3B and 5). In the short term incubation (Fig. 6) GH separated on gel filtration on Sephadex G-50 (superfine) into two peaks (Fig. 9, GH) with the larger molecular weight species identified as peptide 43–169 and the smaller molecular weight species identified as a mixture of peptides 89–169 and 92–169 (Table I and II). These latter two peptides from the early phase digest were identified by NH2-terminal sequence only because they could not be separated by gel filtration.
In order to characterize more fully the effects of brain cathepsin D on BP microheterogeneous components other than component one, BP component three was subjected to both minimal (Fig. 10) and larger (Fig. 11) amounts of enzyme. The chromatographic profiles were similar but not completely identical with those obtained from digests of BP component one. Based on amino acid composition (Table III) and partial sequence (Table IV) of peptides obtained as shown in Figs. 10 and 11. Peak A could be identified as peptide 43-89, Peak B as peptide 43-88, Peak C as peptide 43-91, and Peak D as peptide 1-42.

Although the additional residues of phenylalanine, lysine, and aspartic acid beyond that expected for peptide 43-88 establish that peptide C contains residues 43-91 (Table III), the identification of peptide A as 43-89 is less certain. The phenylalanine content (Table III) of peptide A is slightly higher than that for peptide B or 43-88 and is consistent with the presence of the additional phenylalanine residue at position 89 (2). The limited increase of phenylalanine may be a consequence of the admixture of peptide 43-88 with peptide 43-89, demonstrable by gel electrophoresis at pH 8.8 (data not shown).

Peaks G and H obtained as described in Fig. 10 separated into two peaks on gel filtration on Sephadex G-50 (superfine) (data not shown) giving a profile similar for GH of the digest of component one (Fig. 9). The larger molecular species from both was peptide 43-169. The smaller molecular weight species for G was peptide 89-169 and that for H was peptide 92-169. G and H, obtained as described in Fig. 11, each showed one predominant peak on Sephadex G-50 (superfine), identified as peptide 89-169 in G and peptide 92-169 in H.

The peptides derived from BP microheterogeneous component three were isolated and studied by gel electrophoresis with or without additional treatment with brain cathepsin D. Similar to the situation with component one, this study demonstrated that peptide 1-42 was degraded to form peptide 1-36 and peptide 43-169 was degraded to form peptide 43-88 and another band which is presumably peptides 89-169 and 92-169. Small amounts of Band A or peptide 43-89 could be identified in the digests of peptide 43-169.

**Effects of Pepstatin**—Since pepstatin is known to inhibit cathepsin D from brain (36) and other organs (37), it was of interest to determine if pepstatin had an effect on the initiation or extent of degradation of BP by brain cathepsin D. Because of the difficulty of solubilizing pepstatin, the quantitative relationships may not be totally accurate; however, it was clear that when pepstatin inhibited the degradation of BP by brain cathepsin D it did so by blocking the initial cleavage of BP at residues 42 and 43.

**DISCUSSION**

BP is very susceptible to degradation by a variety of proteolytic enzymes (38), and this property plus the differences in molecular regions which are encephalitogenic in different laboratory animals (16) led to confusion when encephalitogenic sites of BP were being localized. Brain itself contains

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**Table III**

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Peptides</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>2.1 (2)^b</td>
</tr>
<tr>
<td>Histidine</td>
<td>3.0 (3)</td>
</tr>
<tr>
<td>Arginine</td>
<td>4.6 (5)</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>3.0 (3)</td>
</tr>
<tr>
<td>Threonine</td>
<td>2.0 (2)</td>
</tr>
<tr>
<td>Serine</td>
<td>3.8 (4)</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>1.8 (2)</td>
</tr>
<tr>
<td>Proline</td>
<td>1.8 (2)</td>
</tr>
<tr>
<td>Glycine</td>
<td>2.1 (2)</td>
</tr>
<tr>
<td>Alanine</td>
<td>4.9 (3)</td>
</tr>
<tr>
<td>Valine</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Methionine</td>
<td>1 (1)</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Leucine</td>
<td>3.2 (3)</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.8 (1)</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0.9 (1)</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>ND</td>
</tr>
</tbody>
</table>

\^a Letters in the column headings have the same designation as that used for bands seen on gel electrophoresis and peaks present on column chromatography. Numbers in parentheses following the letters indicate position in the bovine BP molecule (2, 3).

\^b The first number was derived by amino acid analysis of the peptide. Numbers in parentheses represent the number of residues based on the known sequence of bovine BP (2, 3).

\^c ND, not determined.

---

**Table IV**

<table>
<thead>
<tr>
<th>NH\textsubscript{2} terminal sequence of fragments of BP component three</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
</tr>
<tr>
<td>B</td>
</tr>
<tr>
<td>C</td>
</tr>
<tr>
<td>D</td>
</tr>
<tr>
<td>E</td>
</tr>
<tr>
<td>G</td>
</tr>
<tr>
<td>H</td>
</tr>
<tr>
<td>GR</td>
</tr>
</tbody>
</table>

\^a Letters have the same designation as that used for bands seen on gel electrophoresis and peaks on column chromatography. Numbers indicate position in the bovine BP molecule (2, 3).
proteolytic activity in the acidic pH range which may play a role in the fragmentation of BP during its isolation (39, 40). Bovine brain cathepsin D was recognized to cleave bovine BP into three peptides (24), but the sites of scission were initially uncertain because the amino acid sequence had not yet been defined (25). These sites were subsequently identified by one group (26) as the two Phe-Phe bonds of bovine BP located at positions 42 and 43 and 88 and 89, leading to the production of BP peptides 1-42, 43-88, and 89-169. Investigations of the effect of liver cathepsin D on BP resulted in different findings, as the Phe-Phe bond at position 42 and 43 was the only apparent site of cleavage giving rise to peptides 1-42 and 43-169. Trace amounts of other peptides were also found with one of these suggested to be, but not positively identified as, peptide 43-88 (3). In studies using brain cathepsin D (26) an extensive degradation of BP was permitted, whereas studies utilizing liver cathepsin D involved less extensive digestion of BP (3). Although a difference in substrate specificity between brain and liver enzymes is possible, our findings suggest that these previously reported differences were a reflection of the extent of the sequential degradation of BP.

The present results indicate that bovine brain cathepsin D degrades BP in a stepwise fashion with a rapid initial cleavage of the Phe-Phe bond at residues 42 and 43 giving rise to peptides 1-42 and 43-169. Although intact BP quickly disappears after the first cleavage, the limited hydrolysis is incomplete, and for further treatment with cathepsin D peptide 42 is degraded to peptide 1-36 and peptide 43-169 is degraded to peptides 43-88, 89-169, 92-169, and small amounts of peptides 43-89. The presence of peptide 43-89 was more obvious in the fragmentation of BP microheterogeneous components two and five than of component one. Peptide 43-91 also transiently appeared, but residues 37 to 42 could not be identified by the methods used. As indicated by the presence of peptides 43-89 and 43-91 as well as the presence of peptide 92-169 in the early phase of digestion, brain cathepsin D is capable of directly cleaving peptide 43-169 at both the Phe-Phe bond at residues 88 and 89 and the Asn-Ile bond at residues 91 and 92. There was no indication that peptide 92-169 was formed from peptide 89-169 by aminopeptidase activity in brain cathepsin D or a contaminating enzyme. Our data do not indicate a simultaneous cleavage of both Phe-Phe bonds. The action of brain cathepsin D on BP shows many pepsin-like features, but peptide 1-88 or peptide 37-88 is not formed by the action of brain cathepsin D as it is by pepsin (10, 18). Furthermore, the Asn-Ile bond at position 91 and 92 and the Leu-Asp bond at position 36 and 37 may also be cleaved. The cleavage of Leu-Asp and Asn-Ile bonds by cathepsin D had not previously been recognized; whereas several other bonds, known to be broken by cathepsin D (20, 41) were not cleaved. The appearance of peptide 43-89 indicates that the Phe-Lys bond at position 89 and 90 may be cleaved and this is not surprising in view of the susceptibility to cleavage of bonds in which an aromatic residue participates (20). The pattern of changes suggests that peptides 43-91 and 92-169 are formed as a result of cleavage of the Asn-Ile bond at position 91 and 92 in peptide 43-169, with the rapid further degradation of peptide 43-91 to peptides 43-88 or 43-89. Brain cathepsin A has been shown to remove the Phe at position 96 from peptide 40-88 (42), but the optimum for that reaction is at pH 5 and the cathepsin D preparation used in this study meets criteria for homogeneity (29). Except for peptide 43-89, there does not seem to be a difference in the peptides formed by degradation of different BP microheterogeneous components, however, charge differences did occur in peptides 43-169, 89-169, and 92-169 consistent with the carboxyl half of the BP molecule containing sites for modification of amino acid residues and alteration of charges (10). The amino acid composition data agreed with others (10) in furnishing no evidence for the loss of COOH-terminal arginine residues from component one to three to also account for microheterogeneity in bovine BP as reported for guinea pig BP (43). The determination of possible differences in the kinetics of the degradation of various BP components and BP peptides by cathepsin D must await a method for clearly separating reaction products from substrate.

The finding that there is a defined order for the attack by brain cathepsin D on the two Phe-Phe bonds in BP implies the influence of other factors, such as conformation, in the molecular regions near these bonds. Importance of the interaction between substrate and enzyme has been demonstrated for the activity of bovine spleen cathepsin D on synthetic oligopeptides (44). High resolution nuclear magnetic resonance spectroscopy of bovine BP indicates molecular folding in the region of residues 84-115 in BP (45), and immunochromatographic studies have demonstrated that the portion of bovine BP containing residues 80 to 88 contains an antigenic determinant which is inaccessible or not present in the intact molecule (46). The demonstration that brain-derived fibroblast growth factor is identical with BP peptides 43-153 and 43-165 while intact BP had no such activity (15) places new emphasis on understanding the degradation products of BP and their biological properties. The appearance of BP peptides in the body fluids of patients with multiple sclerosis (27) may exert a biological, but nonimmunological, effect in perpetuating disease or producing symptoms. The effects of the enzyme on the microheterogeneous components of the molecule in forming fragments of different charge characteristics suggest that the processes regulating microheterogeneity may influence the outcome of degradation of BP by brain cathepsin D and possibly other proteinases.

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REFERENCES

The sequential limited degradation of bovine myelin basic protein by bovine brain cathepsin D.
J N Whitaker and J M Seyer


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