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*CURRENT STATUS OF THE STRUCTURE OF PAPAIN: THE LINEAR SEQUENCE, ACTIVE SULFHYDRYL GROUP, AND THE DISULFIDE BRIDGES**

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Knowledge of the complete amino acid sequence of papain is a necessary prerequisite to an understanding of structure to function relationships and the mechanism of action of this proteolytic enzyme. Furthermore, the structure of papain is of added interest as a model of a proteolytic enzyme displaying a requirement for a free sulfhydryl group. It should be recalled that papain shows those properties typical of a "sulfhydryl enzyme."[1] Investigation of the structure of papain was initiated by us a number of years ago and this effort has involved the collaboration of a number of investigators.[2]

The amino acid composition of papain was derived from recent analyses performed on the Spinco amino acid analyzer. The total number of amino acids is close to 200 which is appreciably higher than the 178 reported in 1954.[3] It will still be necessary to await the completion of the amino acid sequence in order to deduce the exact composition of this protein; the precision of measurement with a protein of this size places a degree of uncertainty on the exact number of residues.

Papain contains a single sulfhydryl group at the active center.[4] This group is partly "masked" in the crystalline enzyme but is present in stoichiometric amounts after activation with mercaptans[4, 5] or sodium borohydride.[5] The activity of

papain preparations before activation is directly related to the reactive sulfhydryl content.[4, 5] The remaining sulfur residues are present in three disulfide bridges.[5]

Prior to structural studies, the enzyme must be inactivated to prevent autolysis and the disulfide bridges cleaved and converted to a stable derivative to prevent disulfide interchange and to increase the susceptibility of the substrate to proteolysis. Performic acid oxidation was first used to convert the sulfur residues to the stable cysteic acid residues,[6] whereas in recent studies we employed reductive cleavage of disulfide bonds with mercaptoethanol in urea and alkylation of the resulting sulfhydryl groups with iodoacetate.[7] These papain derivatives served as substrates for tryptic and chymotryptic hydrolyses.

For identification of the sequence containing the catalytically active sulfhydryl group and the establishment of the position of the disulfide bridges, we have used crystalline papain inactivated with C^{14}-labeled iodoacetate. The sulfhydryl group in the active enzyme was converted to the inactive *S*-carboxymethyl derivative in 53 per cent yield.[8]

Fractionation of peptides was accomplished by chromatography on ion exchange resins, either Dowex-50 or Dowex-1, or a combination of the two. Further purification, when required, was performed by chromatography or electrophoresis on paper, and by chromatography on dextran gels. The purity of isolated peptides was established by a two-dimensional separation on paper with electrophoresis in one direction at pH 1.9 or 6.5, and chromatography in the second with butanol-acetic acid-water or butanol-pyridine-acetic acid-water. After hydrolysis with 6 *N* HCl for 20 or 40 hr, each peptide was analyzed quantitatively by the method of Spackman, Stein, and Moore.[9] The stoichiometry, purity, and yield were determined from the analyses without correction. The yields were variable and ranged from 5 to 70 per cent.

The sequences of peptides were determined by a variety of techniques. The Ed-

TABLE 1

PEPTIDES ISOLATED FROM TRYPTIC DIGESTS

1.	O-T-VI	Gly-Gly-Ileu-Phe-Val-Gly-Pro-CyS-Gly-Asn-Lys
2.	O-T-X	Tyr-CyS-Arg
3.	O-T-XII	Gly-Ala-Val-Thr-Pro-Val-Lys
4.	O-T-XIV	Thr-Asp-Gly-Val-Arg
5.	O-T-XV	Glu-Lys
6.	O-T-XVIII	Asn-Thr-Pro-Tyr-Tyr-Glu-Gly(Val,Gln)Arg
7.	O-T-XXI	Ileu-Lys
8.	O-T-XXII	Ser-Arg
9.	O-T-XXIII	Asp-Phe-Gln-Leu-Tyr-Arg
10.	A-T-XXVI	Gly-Pro-Tyr-Ala-Ala-Lys
11.	A-T-XXVIII	Gln-Lys
12.	A-T-XXIX	(Val,Asp,His,Ala,Val,Ala)Ala(Val,Gly)Tyr(Asn,Pro,Gly)Tyr(Ileu,Leu)-Ileu-Lys
13.	A-T-XXX	Gly-Thr-Gly-Asn-Ser-Tyr-Gly-Val(CyS,Gly)Leu-Tyr(Thr,Ser,Ser,Phe)-Tyr-Pro-Val-Lys-Asn
14.	A-T-XXXI	(Thr,Asn,Gly,Leu,Asn,Asn,Tyr)(Ser,Glu,Gln,Glu,Leu,Leu)(Asp,CyS,Asp)-Arg-Arg
15.	A-T-XXXII	(CyS,Asp,Ser,Glu,Pro,Gly(3),Ala,Leu(2),Tyr,Trp)(Val,Ala,Gln,Tyr)(Gly,Ileu,His,Tyr)Arg
16.	A-T-XXXIII	Ileu-Pro-Glu-Tyr-Val-Asp-Trp-Arg
17.	A-T-XXXIV	Asn-Ser-Trp-Gly-Thr-Gly-Trp-Gly-Glu(Asn,Gly)Tyr-Ileu-Arg
18.	A-T-XXXV	(Gln,Val,Gln,Pro)Tyr-Asn-Gln-Gly-Ala-Leu-Leu-Tyr-Ser-Ileu-Ala-Asn(Gln,Pro,Ser)Val-Val-Leu(Gln,Ala,Ala,Gly)Lys

Performic acid-oxidized papain[11] was employed as substrate in the studies of peptides 1–9, and the *S*-carboxymethyl derivative of papain[12] was used in the studies of peptides 10–18. Peptide 15, A-T-XXXII, was studied in a limited way and the composition of the amino-terminal part should be considered as tentative.

man procedure and leucine aminopeptidase digestion were used to determine the sequence at the amino-terminus. Hydrolysis with carboxypeptidases A and B were employed for studies at the carboxyl-terminus. Complete enzymic digestion of peptides with leucine aminopeptidase with or without prolidase was a useful adjunct to acid hydrolysis to evaluate the amide and tryptophan content. Large peptides were degraded further to smaller fragments by hydrolysis with trypsin, chymotrypsin, papain, or pepsin. The use of these several techniques made it possible to establish the sequence of each peptide.

Results.—The peptides which have led to the establishment of a working hypothesis for the structure of papain are listed in Tables 1, 2, 3, 4, and 5. The nomenclature for the identification of individual peptides is that used in earlier publications.[10, 11] The peptides obtained from tryptic (Table 1) and chymotryptic (Table 2) digests include all those that were studied, whereas the peptides from the peptic digest (Table 4) do not include the disulfide-containing peptides, which are listed

TABLE 2

PEPTIDES ISOLATED FROM CHYMOTRYPTIC DIGESTS

1.	O-C-I	Gly-Val-CyS-Gly-Leu-Tyr
2.	O-C-IV	Asn-Gln-Gly-Ala-Leu-Leu
3.	O-C-V	Thr-Ser-Ser-Phe
4.	O-C-VIII	Asn-Pro-Gly-Tyr
5.	O-C-IX	Gln-Ala-Ala-Gly-Lys-Asp-Phe
6.	O-C-XI	Glu-Gly-Val-Gln-Arg-Tyr
7.	O-C-XIV	Ala-Ala-Lys-Thr(Asp,Gly,Val)Arg-Gln-Val-Gln-Pro-Tyr
8.	O-C-XXA	CyS-Arg-Ser-Arg-Glu-Lys-Gly-Pro-Tyr
9.	O-C-XXB	Arg-Gly-Gly-Ileu-Phe
10.	O-C-XXI	Gly-Ileu-His-Tyr
11.	O-C-XXII	(Val,Gly,Pro,CyS)Gly-Asn-Lys-Val-Asp-His-Ala-Val-Ala-Ala-Val-Gly-Tyr
12.	O-C-XVII,XXIII	Arg-Asn-Thr-Pro-Tyr-Tyr
13.	A-C-XXV	Asp-CyS-Asp-Arg-Arg-Ser-Tyr
14.	D-C-XXX	Ala-Phe
15.	D-C-XXVII	Tyr-Pro-Val-Lys-Asn
16.	D-C-XXVIII	Ser-Glu-Gln-Glu-Leu-Leu
17.	D-C-XXIX	Gly-Thr-Gly-Trp
18.	D-C-XXXII	Ileu-Arg(Ileu,Lys)
19.	D-C-XXXIII	Gly-Glu-Asn-Gly-Tyr
20.	D-C-XXXIV	Ser-Ala-Leu
21.	A-C-XXXV	Ileu-Pro-Glu-Tyr-Val-Asp-Trp
22.	A-C-XXXVI	Ser-Ileu-Ala-Asn-Gln-Pro-Ser-Val-Val-Leu
23.	A-C-XXXVII	(Val,Ala,Gln)Tyr
24.	A-C-XXXVIII	Gly-CyS-Tyr-Pro-Gly-Asp-Gly-Trp
25.	A-C-XXXIX	Ileu-Leu-Ileu-Lys-Asn
26.	A-C-XL	Ileu-Lys-Asn-Ser-Trp
27.	A-C-XLI	Arg-Gly-Thr-Gly-Asn
28.	A-C-XLII	Gln-Leu-Tyr

Peptides 1–12 were derived from studies on performic acid-oxidized papain,[13, 14] peptides 15–18 from denatured papain,[15] and the remaining peptides from the *S*-carboxymethyl derivative.[15]

TABLE 3

PEPTIDES WITH OVERLAPPING SEQUENCES ISOLATED FROM CHYMOTRYPTIC DIGESTS[16]

1. Thr-Ser-Ser-Phe-Tyr-Pro-Val-Lys-Asn
2. Gln-Ala-Ala-Gly-Lys-Asp-Phe-Gln-Leu-Tyr
3. Asn-Gln-Gly-Ala-Leu-Leu-Tyr
4. Tyr-Ser-Ileu-Ala-Asn-Gln-Pro-Ser-Val-Val-Leu
5. Gly-Thr-Gly-Trp-Gly-Glu-Asn-Gly-Tyr
6. Arg-Gly-Gly-Ileu-Phe-Val-Gly-Pro-CyS-Gly-Asn-Lys-Val-Asp-His-Ala-Val-Ala-Ala-Val-Gly-Tyr-Asn-Pro-Gly-Tyr

The yield of peptides 1–6 was low and only limited sequence studies were performed. From these studies and the composition of each, peptides 1–6 include peptides listed in Table 2.

TABLE 4

PEPTIDES ISOLATED FROM PEPTIC DIGESTS[17]

1.	P-I	(Ileu,Pro,Glu)Tyr
2.	P-III	Val-Asp-Trp-Arg-Gln-Lys(Gly,Ala,Val,Thr)
3.	P-IV	Arg-Gln-Lys-Gly(Ala,Val,Thr)
4.	P-V	Arg-Gln(Lys,Gly,Ala,Val,Thr,Pro,Val,Lys,Asn,Gln,Gly,Ser,CyS,Gly, Ser,CyS)
5.	P-VI & P-VII	(Pro,Val,Lys)Asn-Gln-Gly(Ser,CyS,Gly,Ser)CyS-Trp
6.	P-VIII	Tyr-Arg-Gly-Gly-Ileu-Phe
7.	P-IX	(Tyr,Ser,Glu,Gln,Glu,Leu)
8.	P-X	Phe-Gln-Leu
9.	P-XI	Tyr-Ser(Ileu,Ala,Asn,Gln,Pro,Ser,Val,Val)
10.	P-XII	(Val,Arg,Gln,Val,Gln,Pro)Tyr-Asn-Gln
11.	P-XIII	Tyr-Thr-Ser-Ser
12.	P-XIV	Val-Ala-Gln-Tyr(Gly,Ileu,His)
13.	P-XV	(Ileu,Arg)(Ileu,Lys)Thr-Gly-Asn-Leu(Asn,Gln)
14.	P-XVI	Ileu(Lys,Asn,Ser,Trp,Gly,Thr,Gly)
15.	P-XVII	(Ala,Val,Gly,Tyr,Asn,Pro,Gly,Tyr)

Denatured papain with intact disulfide bridges was employed as substrate.

TABLE 5

PEPTIDES ISOLATED AFTER DILUTE ACID HYDROLYSIS[18]

1.	A-H-40b	Gly-Val-Arg-Glu-Val-Glu
2.	A-H-44c	His-Ala-Val-Ala-Ala-Val-Gly-Tyr
3.	A-H-51	Phe-Gln-Leu-Tyr-Arg-Gly-Gly
4.	A-H-69a	Gly(Val,Gln,Arg,Tyr,CyS,Arg,Ser,Arg,Glu,Lys,Gly)
5.	A-H-86	Arg(Arg,Ser,Tyr,Gly)
6.	A-H-69b	Gly-Tyr(Ileu,Arg,Ileu,Lys)
7.	A-H-55	Gln-Tyr-Gly(Ileu,His)Tyr-Arg
8.	A-H-19	Ser-Tyr-Gly-Val-CyS-Gly

Peptides with half-cystine residues were isolated as *S*-carboxymethyl derivatives.

separately, nor the many small fragments produced by extensive degradation of large peptides. No attempt was made to isolate all the peptides present in the peptic digest. A few peptides obtained from partial acid hydrolysates are listed in Table 5.

The structure of the papain molecule, as presently understood, is shown in Figure 1. The alignment of peptides is based on the independent evaluation of the amino-terminal and carboxyl-terminal sequences of papain.[17, 19–21] From the two ends, peptides containing these partial sequences were located. Overlapping sequences of various peptides from two or more digests have permitted a unique alignment of all the known peptides.

The first 26 amino acids from the amino-terminus are shown in a complete sequence.[17] An overlap is required to bridge this first sequence and the next four residues to the central long sequence. Again, a peptide bridge is still needed to overlap peptides near the carboxyl end of the papain molecule. It is apparent that all but seven residues have been placed in very long sequences. The total number of residues placed in sequence is 196. Further, it should be noted that the inclusion

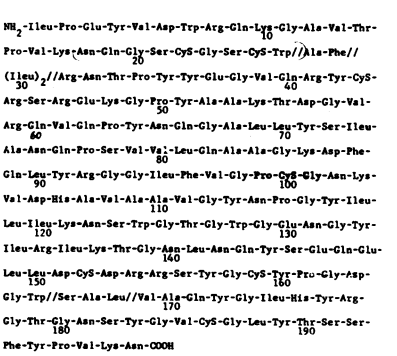

FIG. 1.—Tentative amino acid sequence of papain. Amino acid residues placed in sequence are separated by dashes; slant lines indicate unassigned peptides whose relative positions are not yet established.

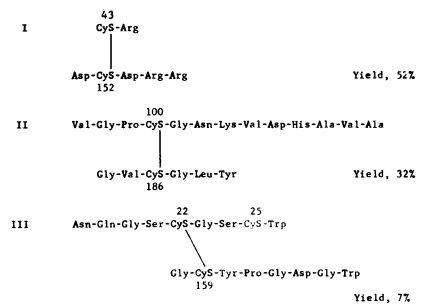

FIG. 2.—Mono-C^{14}-*S*-carboxymethyl-papain was hydrolyzed successively with chymotrypsin, trypsin, and pepsin or by chymotrypsin and trypsin. Tests with the nitroprusside reagent aided in the location of disulfide-containing fractions after Dowex-50 chromatography and on subsequent separation by paper chromatography. Fractions containing C^{14} were detected by counting in a liquid scintillation counter or with a paper strip scanner. The peptides were isolated in purified form, and the compositions were obtained by amino acid analysis before and after converting the sulfur residues to stable derivatives (cysteic acid for peptides I and II, and *S*-aminoethylcysteine for peptide III).

of two isoleucine residues brings the total number of amino acids close to the value found by amino acid analysis. Thus, it is apparent that the structure can serve as a working hypothesis for the complete amino acid sequence of papain.

The assignment of the positions of the disulfide bridges is now possible since each of the seven half-cystine residues has been located (Fig. 1). The mono-*S*-carboxymethyl derivative of papain containing intact disulfide bridges was hydrolyzed successively with chymotrypsin, trypsin, and pepsin.[8] The disulfide-containing peptides were located with the use of the nitroprusside reagent, and the peptide containing C^{14}-carboxymethylcysteine was located by measurements in a liquid scintillation counter. The three peptides listed in Figure 2 were isolated as disulfide-containing peptides. Peptides I and II were present in 30–50 per cent yield and at the same level as other peptides found in the digest. Peptide III containing the C^{14}-carboxymethyl derivative, a disulfide, and two tryptophan residues was isolated in lower yield, presumably because of the chemical reactivity of these residues and because of incomplete hydrolysis.

The assignment of the disulfide bridges to known parts of the sequence was facilitated by oxidation of the disulfide bonds in peptides I and II with performic acid. Each of the peptides containing cysteic acid was isolated and its composition determined. Peptide I contained the sequences $CySO_3H$-Arg and Asp-$CySO_3H$-Asp-Arg-Arg. Peptide II yielded Val-Gly-Pro-$CySO_3H$-Gly-AspNH_2-Lys-Val-Asp-His-Ala-Val-Ala as one fragment and Gly-Val-$CySO_3H$-Gly-Leu-Tyr as the other. From these sequences, it is evident that peptide I contains a disulfide bridge between residues 43 and 152, and peptide II between 100 and 186 (see Figs. 1 and 2).

Peptide III, which contains tryptophan residues, was reduced with mercaptoethanol, and the resulting sulfhydryl groups were converted to the *S*-aminoethyl derivatives by reaction with ethylenimine.[22] The two fragments were isolated by paper electrophoresis. Peptide III-a (Fig. 3) was radioactive and contained one residue each of *S*-carboxymethylcysteine (CMC) and *S*-aminoethylcysteine (AEC), whereas peptide III-b contained the *S*-aminoethyl derivative. It is evident that the active sulfhydryl group is present in the sequence of peptide III-a. Its position in the peptide was established by digestion with trypsin which hydrolyzes at the carboxyl bond of the *S*-aminoethylcysteinyl residue. The two fragments, peptides III-a-1 and III-a-2, were separated, and the radioactivity and the *S*-carboxymethylcysteine were both located in peptide III-a-2. The sequences

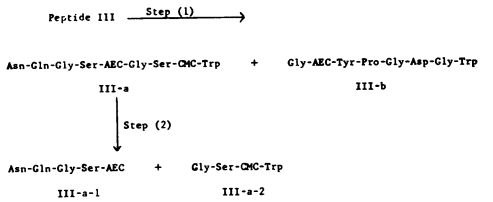

FIG. 3.—The peptide was treated [step (1)] with mercaptoethanol at pH 8 for 20 hr and a five-fold excess of ethyleneimine added. After 1 hr, the sample was lyophilized and purified by paper electrophoresis and then paper chromatography. Peptide III-a was digested with trypsin [step (2)] at pH 8.2, 40°, for 24 hr, and the products were separated by paper electrophoresis. Sequence studies on the various fragments made use of the Edman procedure and enzymic degradation.

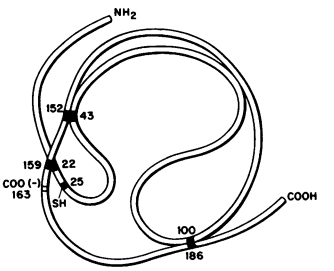

FIG. 4.—A diagramatic representation of the structure of papain, illustrating the relative positions of the disulfide bridges, the active sulfhydryl group, and the aspartyl group at position 163.

shown were established by the Edman degradation. The sequence determination of peptide III-b was based on the Edman degradation and hydrolysis by carboxypeptidase and leucine aminopeptidase.

The position of peptides III-a and III-b in the sequence of papain establishes a disulfide bridge between residues 22 and 159, and the active sulfhydryl group at position 25. The location of the disulfide bridges and the reactive sulfhydryl provides evidence from structural studies for the presence of a single cysteine residue and three disulfide bridges in papain in consonance with investigations by other methods.[5]

Discussion.—The positions of the disulfide bridges in papain places a constraint on the structure of the molecule, that is, the molecule must be folded to accommodate the disulfide bridges. It will be necessary to await detailed X-ray crystallographic studies before the three-dimensional structure becomes known. In the meantime, a two-dimensional structure can be examined based on the position of the disulfide bridges (Fig. 4).

The gross picture of papain is that of a compact molecule which can be observed from the folding back of the chain to form each disulfide bridge. The bridge at 22–159 brings the amino-terminal region in close proximity to a section of the molecule only 39 residues removed from the carboxyl-terminus. The middle region of papain, near residue 100, is very close to the carboxyl-terminus. In this manner, one can visualize the occurrence of strong interactions between side chains along the polypeptide chain producing an extremely compact structure. Such a structure is in accord with optical rotation studies of papain in denaturing systems which showed that only a small change occurred in the transition from the native to the denatured state.[23] The involvement of hydrophobic interactions would contribute to the stability of such a structure. It should be recalled that the spectrophotometric titration of papain showed that 12 phenolic groups were readily ionized, and therefore seven residues were "buried" within the molecule.[24] It is clear that extensive studies are required to define the nature of all the interactions present in papain.

The active sulfhydryl at position 25 is located in a small loop containing approximately 30 amino acid residues. The size of this loop is tentative since this part of the molecule is one of the sections in which overlapping peptides are required. It is possible, therefore, that the number 30 may be revised in future studies by a few

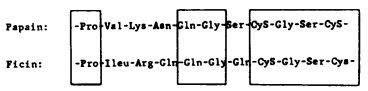

FIG. 5.—The sequences of peptides containing the reactive sulfhydryl groups of papain and ficin. The boxed residues are identical in the two proteolytic enzymes. The papain sequence represents residues 15–25, inclusive. The position of the sequence of ficin is unknown.[25]

residues. Nevertheless, it is possible to draw the obvious conclusion that the position of the active site is in a unique part of the molecule. The environment close to the active sulfhydryl group is of particular interest in any consideration of the properties and function of papain. It should be noted that only two amino acids separate the half-cystine residues at positions 22 and 25, namely, glycine and serine. This close proximity of a sulfhydryl group and a disulfide bridge may facilitate a disulfide interchange between the two. Indeed, a peptide has been isolated in low yield from acid-inactivated papain which contained a disulfide bridge between residues 22 and 25.[17] Thus, one form of inactive papain may be produced by this change in structure.

It is also of interest to compare the sequence around the active site of papain with the sequence of another proteolytic enzyme with similar enzymic properties. Wong and Liener[25] studied the amino acid sequence of a peptide involving the reactive thiol of ficin. In Figure 5, the appropriate sequence in papain is compared to the peptide sequence of ficin. The amino acid residues placed in boxes are identical in both enzymes, whereas the amino acids which differ possess similar properties. The remarkable similarity of the sequences in these two enzymes must have a direct bearing on the function and mechanism of action.

Some years ago it was suggested, on the basis of kinetic studies with papain, that a sulfhydryl group was present at the active site as well as an ionized carboxyl group, probably an aspartyl residue.[26] There is no residue bearing a free carboxyl group in the sequence near the sulfhydryl group; however, there is an aspartyl residue at position 163. Since there is a disulfide bridge between residues 22 and 159, the sulfhydryl group at position 25 is probably in close proximity to the aspartate at position 163—these residues being three and four residues removed from the bridge, respectively. These implications derived from the structure are tentative at this time but serve to draw attention to the necessity for future studies concerned with this aspect of the problem.

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*SELF-SUFFICIENCY OF NATURAL E. COLI POLYSOMES FOR AMINO ACID INCORPORATION**

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In *Escherichia coli*, as in other types of cells, a large proportion of the ribosomes is present in the form of polysomes, as can be shown by analyzing osmotically lysed spheroplasts.[1] The more drastic procedures necessary for the disruption of intact cells, however, easily destroy polysomes, with the resultant appearance of the amino acid incorporating activity in "active" 70s particles.[2] By carefully controlling the temperature and technique, it is nevertheless possible to preserve some heavy amino acid incorporating structures in crude extracts of whole *E. coli* cells disrupted both by alumina grinding[3] and by the French press at relatively low pressures.[4]

Whether in the form of "active" 70s or polysomes, the proportion of ribosomes in extracts of whole cells containing messenger or fragments of mRNA, and participating in amino acid incorporation, does not exceed 10 per cent of the total ribosomes.[2, 5] It seemed therefore of interest to reinvestigate the requirements for amino acid incorporation with isolated, active polysomes as opposed to gross ribosomal preparations consisting largely of inactive 70s particles. A preliminary report of this work has appeared.[6]

Materials and Methods.—The methods used for the preparation of crude extracts