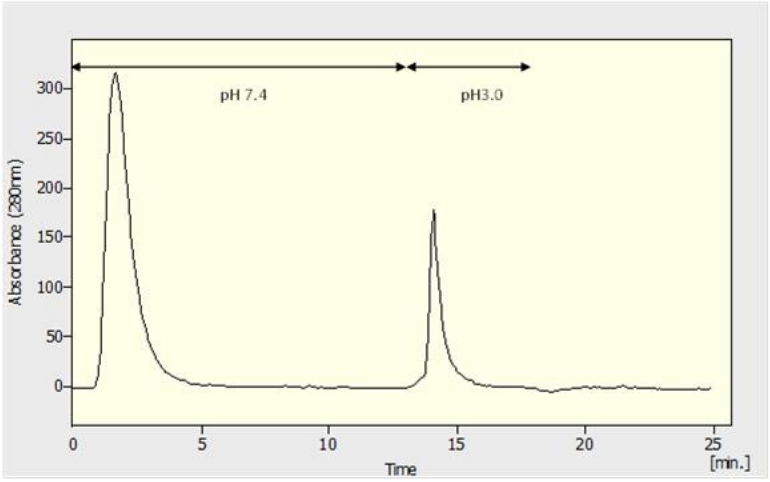
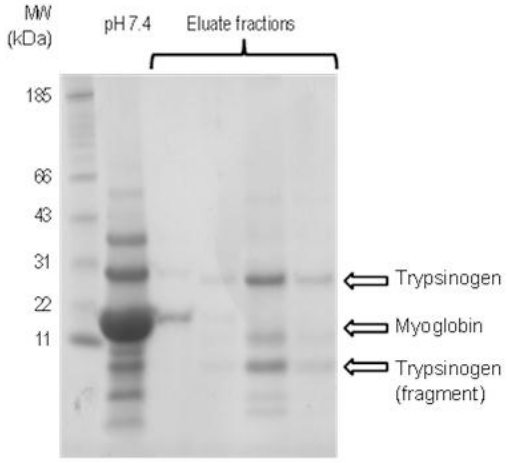
Appendix Figure 1 Selectivity assessment of the employed Benzamidine-Sepharose affinity chromatography

Chromatogram (a) shows fractionation of components in a mixture of model proteins. SDS-PAGE (b) with subsequent component identification, using proteomic procedures, confirmed the selective retention of (the serine protease) bovine trypsinogen and the exclusion of horse myoglobin using the employed mobile phase buffer system.

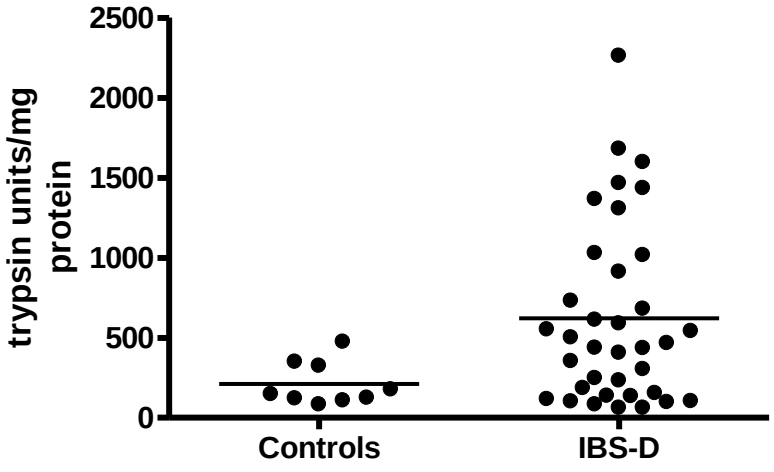
(a)



(b)



Appendix Figure 2 FP in trypsin units/mg protein in 36 IBS-D patients compared to 9 healthy controls showing wide variability in IBS-D values which were significantly greater than controls, p=0.038



Appendix Table 1 Comparison of included and excluded IBS-D patients				
	Gender M/F	Age years Mean (SEM)	Bowel movements/day Median (IQR)	Anxiety HADS Mean (SEM)
79 with complete data	21/56	40.8(1.5)	2.3(1.2-3.4)	10.0(0.6)
49 excluded	15/34	41.8(1.5)	2.6(1.8-4.1)	10.0(0.3)

Legend to Table 1 appendix

There were no significant differences between the groups for any parameter.

Appendix Table 2: Summary of protein structure data determined from the proteomic analysis of Benzamidine-Sepharose enriched proteins, using tandem mass-spectrometry

Name	Sequence coverage	Number of peptides	MOWSE score
Human Pancreatric Alpha-amylase 2A	1 MKFFLLLFTI GFCWAQYSPN TQQGRTSIVH LFEWRWVDIA LECERYLAPK 51 GFGGVQVSPP NENVAIYNPF RPWWERYQPV SYKLCTRSGN EDEFRNMVTR 101 CNNVGVRIYV DAVINHMCGN AVSAGTSSTC GSYFNPGSRD FPAVPYSGWD 151 FNDGKCKTGS GDIENYNDAT QVRDCRLTGL LDLALEKDYV RSKIAEYMNH 201 LIDIGVAGFR LDASKHMWPG DIKAILDKLH NLNSNWFPAG SKPFIYQEVI 251 DLGGEPIKSS DYFGNGRVTE FKYGAKLGTV IRKWNGEKMS YLKNWGEGWG 301 FVPSDRALVF VDNHDNQRGH GAGGASILTF WDARLYKMAV GFMLAHPYGF 351 TRVMSSYRWP RQFQNGNDVN DWVGPPNNNG VIKEVTINPD TTCGNDWVCE 401 HRWRQIRNMV IFRNVVDGQP FTNWYDNGSN QVAFGRGNRG FIVFNNDDWS 451 FSLTLQTGLP AGTYCDVISG DKINGNCTGI KIYVSDDGKA HFSISNSAED 501 PFIAIHAESK L	60	2012

Human Alpha-amylase 2B	1 MKFFLLLFTI GFCWAQYSPN TQQGRTSIVH LFEWRWVDIA LECERYLAPK 51 GFGGVQVSPP NENVAIHNPF RPWWERYQPV SYKLCTRSGN EDEFRNMVTR 101 CNNVGVRIYV DAVINHMSGN AVSAGTSSTC GSYFNPGSRD FPAVPYSGWD 151 FNDGKCKTGS GDIENYNDAT QVRDCRLVGL LDLALEKDYV RSKIAEYMNH 201 LIDIGVAGFR LDASKHMWPG DIKAILDKLH NLNSNWFPAG SKPFIYQEVI 251 DLGGEPIKSS DYFGNGRVTE FKYGAKLGTV IRKWNGEKMS YLKNWGEGWG 301 FMPSDRALVF VDNHDNQRGH GAGGASILTF WDARLYKMAV GFMLAHPYGF 351 TRVMSSYRWP RQFQNGNDVN DWVGPPNNNG VIKEVTINPD TTCGNDWVCE 401 HRWRQIRNMV NFRNVVDGQP FTNWYDNGSN QVAFGRGNRG FIVFNNDDWT 451 FSLTLQTGLP AGTYCDVISG DKINGNCTGI KIYVSDDGKA HFSISNSAED 501 PFIAIHAESK L	43	1636
Human Trypsin-1 (Trypsinogen I)	1 MNPLLILTFV AAALAAPFDD DDKIVGGYNC EENSVPYQVS LNSGYHFCGG 51 SLINEQWVVS AGHCYKSRIQ VRLGEHNIEV LEGNEQFINA AKIIRHPQYD 101 RKTLNNDIML IKLSSRAVIN ARVSTISLPT APPATGTKCL ISGWGNTASS 151 GADYPDELQC LDAPVLSQAK CEASYPGKIT SNMFCVGFLE GGKDSCQGDS 201 GGPVVCNGQL QGVVSWGDGC AQKNKPGVYT KVYNYVKWIK NTIAANS	20	526
Human Trypsin-2 (Trypsinogen II)	1 MNLLLILTFV AAAVAAPFDD DDKIVGGYIC EENSVPYQVS LNSGYHFCGG 51 SLISEQWVVS AGHCYKSRIQ VRLGEHNIEV LEGNEQFINA AKIIRHPKYN 101 SRTLDNDILL IKLSSPAVIN SRVSAISLPT APPAAGTESL ISGWGNTLSS 151 GADYPDELQC LDAPVLSQAE CEASYPGKIT NNMFCVGFLE GGKDSCQGDS 201 GGPVVSNGEL QGIVSWGYGC AQKNRPGVYT KVYNYVDWIK DTIAANS	24	617

Human Trypsin-3/4 (Trypsinogen III/IV)	1 MCGPDDRCPA RWPGPGRAVK CGKGLAAARP GRVERGGAQR GGAGLELHPL 51 LGGRTWRAAR DADGCEALGT VAVPFDDDDK IVGGYTCEEN SLPYQVSLNS 101 GSHFCGGSLI SEQWVVSAAH CYKTRIQVRL GEHNIKVLEG NEQFINAAKI 151 IRHPKYNRDT LDNDIMLIKL SSPAVINARV STISLPTTPP AAGTECLISG 201 WGNTLSFGAD YPDELKCLDA PVLTQAECKA SYPGKITNSM FCVGFLEGGK 251 DSCQRDSGGP VVCNGQLQGV VSWGHGCAWK NRPGVYTKVY NYVDWIKDTI 301 AANS	8	275
Human Chymotrypsin-like elastase family member 3B	1 MMLRLLSSLL LVAVASGYGP PSSRPSSRVV NGEDAVPYSW PWQVSLQYEK 51 SGSFYHTCGG SLIAPDWVVT AGHCISSSRT YQVVLGEYDR AVKEGPEQVI 101 PINSGDLFVH PLWNRSCVAC GNDIALIKLS RSAQLGDAVQ LASLPPAGDI 151 LPNETPCYIT GWGRLYTNGP LPDKLQEALL PVVDYEHCSR WNWWGSSVKK 201 TMVCAGGDIR SGCNGDSGGP LNCPTEDGGW QVHGVTSFVS AFGCNTRRKP 251 TVFTRVSAFI DWIEETIASH	13	393
Human Carboxypeptidase B	1 MLALLVLVTV ALASAHHGGE HFEGEKVFRV NVEDENHINI IRELASTTQI 51 DFWKPDSVTQ IKPHSTVDFR VKAEDTVTVE NVLKQNELQY KVLISNLRNV 101 VEAQFDSRVR ATGHSYEKYN KWETIEAWTQ QVATENPALI SRSVIGTTFE 151 GRAIYLLKVG KAGQNKPAIF MDCGFHAREW ISPAFCQWFV REAVRTYGRE 201 IQVTELLDKL DFYVLPVLNI DGYIYTWTKS RFWRKTRSTH TGSSCIGTDP 251 NRNFDAGWCE IGASRNPCDE TYCGPAAESE KETKALADFI RNKLSSIKAY 301 LTIHSYSQMM IYPYSYAYKL GENNAELNAL AKATVKELAS LHGTKYTYGP 351 GATTIYPAAG GSDDWAYDQG IRYSFTFELR DTGRYGFLLP ESQIRATCEE 401 TFLAIKYVAS YVLEHLY	5	155
Human Alpha-1-antitrypsin	1 MPSSVSWGIL LLAGLCCLVP VSLAEDPQGD AAQKTDTSHH DQDHPTFNKI 51 TPNLAEFAFS LYRQLAHQSN STNIFFSPVS IATAFAMLSL GTKADTHDEI 101 LEGLNFNLTE IPEAQIHEGF QELLRTLNQP DSQLQLTTGN GLFLSEGLKL 151 VDKFLEDVKK LYHSEAFTVN FGDTEEAKKQ INDYVEKGTQ GKIVDLVKEL 201 DRDTVFALVN YIFFKGKWER PFEVKDTEEE DFHVDQVTTV KVPMMKRLGM 251 FNIQHCKKLS SWVLLMKYLG NATAIFFLPD EGKLQHLENE LTHDIITKFL 301 ENEDRRSASL HLPKLSITGT YDLKSVLGQL GITKVFSNGA DLSGVTEEAP 351 LKLSKAVHKA VLTIDEKGTE AAGAMFLEAI PMSIPPEVKF NKPFVFLMIE 401 QNTKSPLFMG KVVNPTQK	5	173

Appendix Table 2 shows a summary of the proteomics results derived from Swiss-Prot database interrogated MASCOT search engine reports. Total precursor and product ion mass-spectrometry data derived from analyses of trypsinolytic peptides of components excised from SDS-PAGE gel profiles of Benzamidine-Sepharose enriched fractions of patient faeces extracts. The eight most abundant components are listed and the primary sequence detected is shown in bold red, together with the number of peptide identifications with statistically significant scores and the total molecular weight search engine (MOWSE) scores obtained for the components.

Appendix Figure 3:

Multiple sequence alignment of various trypsin sequences

```
TRY1_HUMAN      ----------------------------------------------MNPLLIL-------
TRY2_HUMAN      ----------------------------------------------MNLLLIL-------
TRY3_HUMAN      MCGPDDRCPARWPGPGRAVKCGKGLAAARPGRVERGGAQRGGAGLELHPLLGGRTWRAAR
TRYP_PIG        ------------------------------------------------------------


TRY1_HUMAN      ----TFVAAALAAPFDDDDKIVGGYNCEENSVPYQVSLNSGYHFCGGSLINEQWVVSAGH
TRY2_HUMAN      ----TFVAAAVAAPFDDDDKIVGGYICEENSVPYQVSLNSGYHFCGGSLISEQWVVSAGH
TRY3_HUMAN      DADGCEALGTVAVPFDDDDKIVGGYTCEENSLPYQVSLNSGSHFCGGSLISEQWVVSAAH
TRYP_PIG        ------------FPTDDDDKIVGGYTCAANSIPYQVSLNSGSHFCGGSLINSQWVVSAAH
                            * ********** *  **:********* ********..******.*


TRY1_HUMAN      CYKSRIQVRLGEHNIEVLEGNEQFINAAKIIRHPQYDRKTLNNDIMLIKLSSRAVINARV
TRY2_HUMAN      CYKSRIQVRLGEHNIEVLEGNEQFINAAKIIRHPKYNSRTLDNDILLIKLSSPAVINSRV
TRY3_HUMAN      CYKTRIQVRLGEHNIKVLEGNEQFINAAKIIRHPKYNRDTLDNDIMLIKLSSPAVINARV
TRYP_PIG        CYKSRIQVRLGEHNIDVLEGNEQFINAAKIITHPNFNGNTLDNDIMLIKLSSPATLNSRV
                ***:***********.*************** **:::  **:***:****** *.:*:**


TRY1_HUMAN      STISLPTAPPATGTKCLISGWGNTASSGADYPDELQCLDAPVLSQAKCEASYPGKITSNM
TRY2_HUMAN      SAISLPTAPPAAGTESLISGWGNTLSSGADYPDELQCLDAPVLSQAECEASYPGKITNNM
TRY3_HUMAN      STISLPTTPPAAGTECLISGWGNTLSFGADYPDELKCLDAPVLTQAECKASYPGKITNSM
TRYP_PIG        ATVSLPRSCAAAGTECLISGWGNTKSSGSSYPSLLQCLKAPVLSDSSCKSSYPGQITGNM
                :::*** :  *:**:.******** * *:.**. *:**.****:::.*::****:** .*


TRY1_HUMAN      FCVGFLEGGKDSCQGDSGGPVVCNGQLQGVVSWGDGCAQKNKPGVYTKVYNYVKWIKNTI
TRY2_HUMAN      FCVGFLEGGKDSCQGDSGGPVVSNGELQGIVSWGYGCAQKNRPGVYTKVYNYVDWIKDTI
TRY3_HUMAN      FCVGFLEGGKDSCQRDSGGPVVCNGQLQGVVSWGHGCAWKNRPGVYTKVYNYVDWIKDTI
TRYP_PIG        ICVGFLEGGKDSCQGDSGGPVVCNGQLQGIVSWGYGCAQKNKPGVYTKVCNYVNWIQQTI
                :************* *******.**:***:**** *** **:******* ***.**::**
```

```
TRY1_HUMAN      AANS
TRY2_HUMAN      AANS
TRY3_HUMAN      AANS
TRYP_PIG        AAN-
                ***
```

Multiple sequence alignment (CLUSTAL Omega) of human trypsin sequences (TRY1-TRY3) and porcine trypsin (TRYP_PIG). Asterisks indicate conserved residues in all sequences. Peptides identified by LC-MS/MS analysis of purified Benzamidine-Sepharose enriched components from faecal extracts are highlighted in red. Parent protein sequences from SWISS-PROT; data for TRYP_PIG represents peptides derived from the sequencing grade trypsin used as part of the proteomic identification i.e. exogenous to the purified fractions. Unique peptide sequences exclusively found in only one of the trypsin sequences are <u>underlined</u>. Shaded residues denote tryptic cleavage sites. Note that none of the peptide sequences identified for TRYP_PIG were common to the human sequences, and *vice versa*. *F*urther, for each of the human trypsin sequences unique peptides could be identified.

Appendix Figure 4:

Multiple sequence alignment of various human trypsin and tryptase sequences

```
TRY1_HUMAN      ------------------------------------------------------MNP
TRY2_HUMAN      ------------------------------------------------------MNL

TRY3_HUMAN      MCGPDDRCPARWPGPGRAVKCGKGLAAARPGRVERGGAQRGGAGLELHPLLGGRTWRAAR

TRYB1_HUMAN     ------------------------------------------------------MLNLLL

TRYB2_HUMAN     ---------------------------------------------MLLLAPQMLNLLL

TRYD_HUMAN      ---------------------------------------------MLLLAPQMLSLLL



TRY1_HUMAN      LLILTFVAAALAAPFD----DDDKIVGGYNCEENSVPYQVSLNSG----YHFCGGSLINE

TRY2_HUMAN      LLILTFVAAAVAAPFD----DDDKIVGGYICEENSVPYQVSLNSG----YHFCGGSLISE

TRY3_HUMAN      DADGCEALGTVAVPFD----DDDKIVGGYTCEENSLPYQVSLNSG----SHFCGGSLISE

TRYB1_HUMAN     LALPVLASRAYAAPAPGQALQRVGIVGGQEAPRSKWPWQVSLRVHGPYWMHFCGGSLIHP

TRYB2_HUMAN     LALPVLASRAYAAPAPGQALQRVGIVGGQEAPRSKWPWQVSLRVHGPYWMHFCGGSLIHP

TRYD_HUMAN      LALPVLASPAYVAPAPGQALQQTGIVGGQEAPRSKWPWQVSLRVRGPYWMHFCGGSLIHP

                      .  : ..*      :   ****  . ... *:****.       ********



TRY1_HUMAN      QWVVSAGHCYKSRIQVRLG---EHNIEVLEGNEQFINAAKIIRHPQYDRKTLNNDIMLIK

TRY2_HUMAN      QWVVSAGHCYKSRIQVRLG---EHNIEVLEGNEQFINAAKIIRHPKYNSRTLDNDILLIK

TRY3_HUMAN      QWVVSAAHCYKTRIQVRLG---EHNIKVLEGNEQFINAAKIIRHPKYNRDTLDNDIMLIK

TRYB1_HUMAN     QWVLTAAHCVGPDVKDLAALRVQLREQHLYYQDQLLPVSRIIVHPQFYTAQIGADIALLE

TRYB2_HUMAN     QWVLTAAHCVGPDVKDLAALRVQLREQHLYYQDQLLPVSRIIVHPQFYTAQIGADIALLE

TRYD_HUMAN      QWVLTAAHCVEPDIKDLAALRVQLREQHLYYQDQLLPVSRIIVHPQFYIIQTGADIALLE

                ***::*.**  . ::   .   : . : *  ::*:: .::** **::     . ** *::



TRY1_HUMAN      LSSRAVINARVSTISLPTAPP--ATGTKCLISGWGNTASSG-ADYPDELQCLDAPVLSQA

TRY2_HUMAN      LSSPAVINSRVSAISLPTAPP--AAGTESLISGWGNTLSSG-ADYPDELQCLDAPVLSQA

TRY3_HUMAN      LSSPAVINARVSTISLPTTPP--AAGTECLISGWGNTLSFG-ADYPDELKCLDAPVLTQA

TRYB1_HUMAN     LEEPVNVSSHVHTVTLPPASETFPPGMPCWVTGWGDVDNDERLPPPFPLKQVKVPIMENH

TRYB2_HUMAN     LEEPVKVSSHVHTVTLPPASETFPPGMPCWVTGWGDVDNDERLPPPFPLKQVKVPIMENH
```

```
TRYD_HUMAN      LEEPVNISSHIHTVTLPPASETFPPGMPCWVTGWGDVDNNVHLPPPYPLKEVEVPVVENH
                *.. . :.::: :::**.:.   ..*  . ::***:. .      *  *: :..*:: :

TRY1_HUMAN      KCEASYPG---------KITSNMFCVGFLEGGKDSCQGDSGGPVVCNGQ----LQGVVSW
TRY2_HUMAN      ECEASYPG---------KITNNMFCVGFLEGGKDSCQGDSGGPVVSNGE----LQGIVSW
TRY3_HUMAN      ECKASYPG---------KITNSMFCVGFLEGGKDSCQRDSGGPVVCNGQ----LQGVVSW
TRYB1_HUMAN     ICDAKYHLGAYTGDDVRIVRDDMLCAGNTR--RDSCQGDSGGPLVCKVNGTWLQAGVVSW
TRYB2_HUMAN     ICDAKYHLGAYTGDDVRIVRDDMLCAGNTR--RDSCQGDSGGPLVCKVNGTWLQAGVVSW
TRYD_HUMAN      LCNAEYHTGLHTGHSFQIVRDDMLCAGSEN--HDSCQGDSGGPLVCKVNGT---------
                 *.*.*            : ..*:*.*  .  :**** *****:*.: :

TRY1_HUMAN      GDGCAQKNKPGVYTKVYNYVKWIKNTIAANS
TRY2_HUMAN      GYGCAQKNRPGVYTKVYNYVDWIKDTIAANS
TRY3_HUMAN      GHGCAWKNRPGVYTKVYNYVDWIKDTIAANS
TRYB1_HUMAN     GEGCAQPNRPGIYTRVTYYLDWIHHYVPKKP
TRYB2_HUMAN     GEGCAQPNRPGIYTRVTYYLDWIHHYVPKKP
TRYD_HUMAN      --------------------------------
```

Multiple sequence alignment (CLUSTAL Omega) of human trypsin sequences (TRY1-TRY3) and human tryptases beta1, beta2 and delta (TRYB1_HUMAN, TRYB2_HUMAN and TRYD_HUMAN). Asterisks indicate conserved residues in all sequences. Peptides identified by LC-MS/MS analysis of purified Benzamidine-Sepharose enriched components from faecal extracts are highlighted in red. Note that none of the peptide sequences identified for the human trypsin sequences are present in tryptase alpha/beta-1, tryptase beta-2 or tryptase delta.